PROTOCOLS AND APPLICATIONS GUIDE

PROMEGA

SECOND EDITION

59

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Cover illustration by John Andrews

Promega Protocols and Applications Guide

Second Edition

Part Number Y981

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Promega Protocols and Applications Guide

The *Promega Protocols and Applications Guide* is intended to be a companion to the Promega *Catalogue* and a useful reference book for the molecular biology laboratory. This guide may be used in conjunction with the catalogue to learn more about Promega products, or at the lab bench as a convenient source of detailed reference information and step-by-step protocols for the use of these products.

The second edition of the *Promega Protocols and Applications Guide* has been updated and expanded and several new protocols have been added. Significant additions include:

- protocols for protein structural analysis and sequencing
- properties and applications of cytokines, lymphokines and protein kinases
- strategies and protocols for studies of eukaryotic gene regulation
- an expanded Restriction Enzyme chapter and Technical Appendix

References relevant to the protocols are listed at the end of each chapter. Recipes for the required reagents are provided at the end of each chapter subsection.

We hope you find the *Promega Protocols and Applications Guide* easy to use at the lab bench. Every effort has been made to provide accurate and up-to-date protocols and information. Please note, however, that protocols for the use of Promega products are periodically updated in our Technical Bulletins and Technical Manuals. Also, some extremely detailed information (such as the complete sequences of vectors) has not been included in this guide. For these reasons, we recommend that you refer also to the additional technical literature listed at the end of each chapter. This literature includes relevant Technical Bulletins and Technical Manuals as well as articles published in *Promega Notes*. All of these materials are available upon request from Promega or your Promega distributor.

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David E. Titus, Ph.D.
Editor
March, 1991

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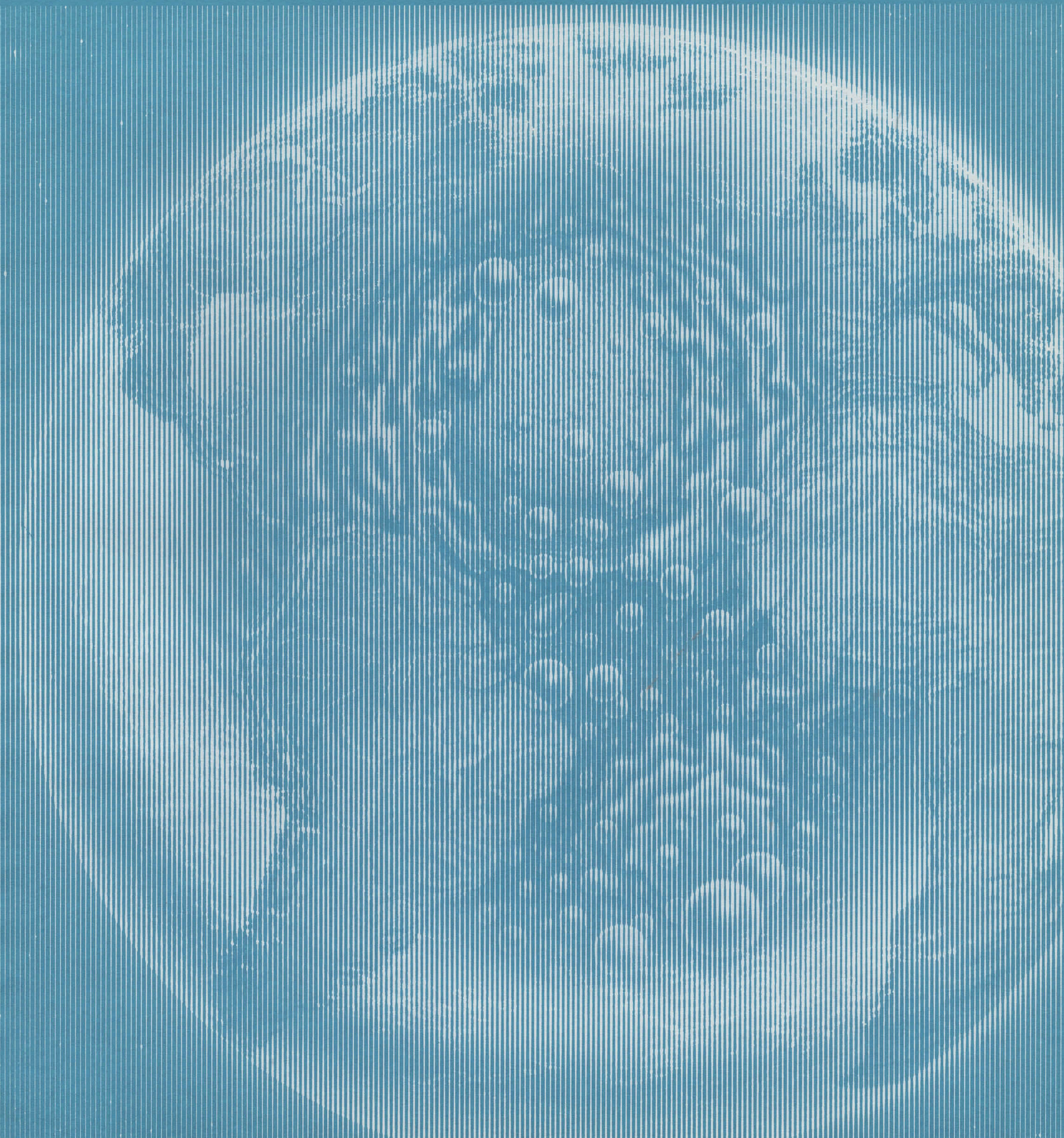

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Restriction Enzymes and Linkers

Table 1. Relative Activity of Restriction Enzymes in Promega's 10X Buffers.

The 10X buffer supplied with each restriction enzyme is optimized to give 100% activity. In many cases, good activity is also obtained using one of Promega's 4-CORE™ 10X buffers. This table may be used to select the best buffer for digestions with multiple restriction enzymes. Enzyme activity is expressed as a percent of the activity obtained with the optimized buffer for each enzyme. Refer to Table 2 for the composition of each restriction enzyme 10X buffer.

Promega Enzyme	Buffer Supplied with Enzyme	Enzyme Activity in the Promega 4-CORE™ Buffers				Enzyme Assay Temp-erature
		A	B	C	D	
Aat II	J	50-75%	10-25%	<10%	<10%	37°C
Acc I	A	100%	75-100%	75-100%	25-50%	37°C
Acc III	F	<10%	10-25%	25-50%	25-50%	67°C
Acy I	F	10-25%	50-75%	50-75%	50-75%	37°C
Alu I	B	75-100%	100%	75-100%	10-25%	37°C
*Alw*26 I	C	10-25%	25-50%	100%	10-25%	37°C
*Alw*44 I	C	<10%	25-50%	100%	25-50%	37°C
Apa I	A	100%	50-75%	50-75%	<10%	37°C
Asu I	B	25-50%	100%	50-75%	10-25%	37°C
Ava I	B	10-25%	100%	50-75%	25-50%	37°C
Ava II	C	50-75%	50-75%	100%	25-50%	37°C
Bal I	G	10-25%	<10%	<10%	<10%	25°C
*Bam*H I	C	75-100%	75-100%	100%	50-75%	37°C
Ban II	B	75-100%	100%	75-100%	25-50%	37°C
Bbu I	A	100%	75-100%	75-100%	<10%	37°C
Bcl I	C	10-25%	75-100%	100%	50-75%	50°C
Bgl I	D	10-25%	25-50%	75-100%	100%	37°C
Bgl II	D	25-50%	75-100%	75-100%	100%	37°C
*Bsp*1286 I	A	100%	50-75%	25-50%	10-25%	37°C
*Bss*H II	B	75-100%	100%	75-100%	50-75%	50°C
*Bst*71 I	D	10-25%	25-50%	25-50%	100%	50°C
*Bst*E II	D	25-50%	50-75%	50-75%	100%	60°C
*Bst*X I	D	<10%	10-25%	25-50%	100%	50°C
*Bst*Z I	L	<10%	<10%	10-25%	75-100%	50°C
*Bsu*36 I	E	<10%	25-50%	50-75%	25-50%	37°C
Cfo I	B	75-100%	100%	75-100%	25-50%	37°C
Cla I	C	75-100%	75-100%	100%	75-100%	37°C

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Promega Enzyme	Buffer Supplied with Enzyme	Enzyme Activity in the Promega 4-CORE™ Buffers A	B	C	D	Enzyme Assay Temperature
Csp I	K	<10%	10-25%	25-50%	50-75%	30°C
*Csp*45 I	B	25-50%	100%	50-75%	25-50%	37°C
Dde I	D	25-50%	25-50%	50-75%	100%	37°C
Dpn I	B	50-75%	100%	75-100%	50-75%	37°C
Dra I	B	75-100%	100%	75-100%	50-75%	37°C
*Eco*47 III	D	<10%	25-50%	50-75%	100%	37°C
*Eco*52 I	L	<10%	<10%	10-25%	25-50%	37°C
*Eco*R I	H	25-50%	50-75%	50-75%	50-75%	37°C
*Eco*R V	D	10-25%	25-50%	50-75%	100%	37°C
Fok I	B	75-100%	100%	75-100%	25-50%	37°C
Hae II	B	50-75%	100%	50-75%	10-25%	37°C
Hae III	C	75-100%	75-100%	100%	50-75%	37°C
Hha I	C	50-75%	75-100%	100%	50-75%	37°C
Hinc II	B	25-50%	100%	25-50%	50-75%	37°C
Hind III	B	25-50%	100%	75-100%	10-25%	37°C
Hinf I	B	50-75%	100%	75-100%	75-100%	37°C
Hpa I	B	50-75%	100%	50-75%	10-25%	37°C
Hpa II	A	100%	50-75%	50-75%	10-25%	37°C
Kpn I	A	100%	25-50%	25-50%	<10%	37°C
Mbo I	C	10-25%	75-100%	100%	50-75%	37°C
Mbo II	B	10-25%	100%	50-75%	75-100%	37°C
Mlu I	D	10-25%	25-50%	50-75%	100%	37°C
Msp I	B	75-100%	100%	75-100%	25-50%	37°C
Nco I	D	50-75%	75-100%	75-100%	100%	37°C
Nhe I	B	75-100%	100%	75-100%	10-25%	37°C
Nar I	G	75-100%	50-75%	75-100%	25-50%	37°C
Not I	D	<10%	10-25%	25-50%	100%	37°C
Nsi I	D	10-25%	50-75%	50-75%	100%	37°C
Pst I	H	10-25%	50-75%	50-75%	50-75%	37°C
Pvu I	D	10-25%	25-50%	50-75%	100%	37°C
Pvu II	B	25-50%	100%	50-75%	25-50%	37°C
Rsa I	C	75-100%	75-100%	100%	<10%	37°C

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Restriction Enzymes and Linkers

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Table 1. Relative Activity of Restriction Enzymes in Promega's 10X Buffers.

Promega Enzyme	Buffer Supplied with Enzyme	Enzyme Activity in the Promega 4-CORE™ Buffers A	B	C	D	Enzyme Assay Temperature
Sac I	A	100%	25-50%	25-50%	<10%	37°C
Sac II	A	100%	50-75%	50-75%	50-75%	37°C
Sal I	D	<10%	10-25%	25-50%	100%	37°C
*Sau*3A I	B	25-50%	100%	75-100%	<10%	37°C
*Sau*96 I	C	25-50%	25-50%	100%	50-75%	37°C
Sca I	K	<10%	>100%[a]	50-75%	75-100%	50°C
Sfi I	B	75-100%	100%	75-100%	25-50%	37°C
Sin I	A	100%	75-100%	50-75%	10-25%	25°C
Sma I	J	<10%	<10%	<10%	<10%	37°C
*Sna*B I	B	50-75%	100%	50-75%	<10%	37°C
Spe I	B	75-100%	100%	75-100%	75-100%	37°C
Sph I	K	75-100%	75-100%	>100%[a]	75-100%	37°C
Spo I	J	75-100%	50-75%	50-75%	10-25%	37°C
Ssp I	E	10-25%	50-75%	50-75%	75-100%	37°C
Stu I	B	75-100%	100%	75-100%	50-75%	37°C
Sty I	F	25-50%	75-100%	75-100%	75-100%	65°C
Taq I	F	10-25%	25-50%	50-75%	50-75%	65°C
*Tth*111 I	B	50-75%	100%	75-100%	25-50%	37°C
Vsp I	D	<10%	25-50%	75-100%	100%	37°C
Xba I	D	50-75%	75-100%	75-100%	100%	37°C
Xho I	D	25-50%	75-100%	75-100%	100%	37°C
Xho II	C	25-50%	25-50%	100%	10-25%	37°C
Xma I	B	50-75%	100%	25-50%	<10%	37°C

Note:

a. Not recommended due to star activity.

Restriction Enzymes and Linkers

Table 2. Composition of Promega Restriction Enzyme Buffers (1X).

Restriction enzyme buffers are provided as 10X stocks. The concentrations listed below are for 1X concentrations. Refer to Table 1 for a list of the 10X buffer and assay temperature used for each restriction enzyme.

Restriction Enzyme 10X Buffer	NaCl	KCl	Tris–HCl	Assay pH and Temperature	$MgCl_2$	DTT
A	6mM	—	6mM	pH 7.5, 37°C	6mM	1mM
B	50mM	—	6mM	pH 7.5, 37°C (pH 7.2 at 50°C) (pH 7.0 at 65°C)	6mM	1mM
C	50mM	—	10mM	pH 7.9, 37°C (pH 7.6 at 50°C)	10mM	1mM
D	150mM	—	6mM	pH 7.9, 37°C (pH 7.6 at 50°C) (pH 7.3 at 60°C)	6mM	1mM
E	100mM	—	6mM	pH 7.5, 37°C	6mM	1mM
F	100mM	—	10mM	pH 8.5, 37°C (pH 7.8 at 65°C)	10mM	1mM
G	—	—	50mM	pH 8.2, 37°C (pH 8.5 at 25°C)	5mM	—
H	50mM	—	90mM	pH 7.5, 37°C	10mM	—
J	—	50mM	10mM	pH 7.5, 37°C (pH 7.8 at 25°C)	7mM	1mM
K	—	150mM	10mM	pH 7.4, 37°C (pH 7.6 at 30°C)	10mM	—
L	100mM	—	10mM	pH 9.0, 37°C	3mM	—

Restriction Enzymes and Linkers

Table 3. Inactivation of Restriction Enzymes by Heat.

Promega Enzyme	Heat Inactivated	Promega Enzyme	Heat Inactivated	Promega Enzyme	Heat Inactivated
Aat II	+	*Csp* I	+	*Pvu* I	–
Acc I	–	*Csp*45 I	+	*Pvu* II	+/–
Acc III	–	*Dde* I	+/–	*Rsa* I	+
Acy I	+	*Dpn* I	+	*Sac* I	+
Alu I	+	*Dra* I	+	*Sac* II	+
*Alw*26 I	+	*Eco*47 III	+	*Sal* I	–
*Alw*44 I	+	*Eco*52 I	+	*Sau*3A I	+
Apa I	+	*Eco*R I	+	*Sau*96 I	–
Asu I	+	*Eco*R V	+	*Sca* I	+
Ava I	+/–	*Fok* I	+	*Sfi* I	+/–
Ava II	+	*Hae* II	–	*Sin* I	+
Bal I	+	*Hae* III	–	*Sma* I	+/–
*Bam*H I	+/–	*Hha* I	+	*Sna*B I	–
Ban II	+	*Hinc* II	+	*Spe* I	+
Bbu I	+	*Hind* III	+/–	*Sph* I	+
Bcl I	–	*Hinf* I	–	*Spo* I	+
Bgl I	+	*Hpa* I	–	*Ssp* I	+
Bgl II	–	*Hpa* II	–	*Stu* I	+
*Bsp*1286 I	+	*Kpn* I	+/–	*Sty* I	+
*Bss*H II	–	*Mlu* I	+/–	*Taq* I	–
*Bst*71 I	–	*Msp* I	+	*Tth*111 I	–
*Bst*E II	–	*Nar* I	+	*Vsp* I	+
*Bst*X I	+/–	*Nco* I	+	*Xba* I	+
*Bst*Z I	–	*Nhe* I	+	*Xho* I	+
*Bsu*36 I	–	*Not* I	+	*Xho* II	+
Cfo I	+/–	*Nsi* I	+/–	*Xma* I	+
Cla I	+	*Pst* I	+/–		

Heat Inactivation Key:

+ = 95% inactivation by incubation at 65°C for 15 minutes.
– = no inactivation.
+/– = partial inactivation.

Notes:

a. 50µl volumes were used.

b. All restriction enzymes listed are manufactured by Promega.

Restriction Enzymes and Linkers

Table 4. NaCl Concentration Effects on Restriction Enzyme Activities.

	NaCl Concentration (mM)			
Enzyme	0	50	100	150
Aat **II**[a]	–	+	+	–
Acc **I**	++	++	–	–
Acc **III**	+	+	++	++
Acy **I**	–	+	++	++
Aha III	–	++	++	++
***Alw*26 I**	+	++	+	+
***Alw*44 I**	–	++	+	+
Alu **I**	–	++	++	–
Apa **I**	++	++	+	–
Asu **I**	–	++	++	–
Ava **I**	++	++	++	++
Ava **II**	++	++	+	–
Bal **I**	++	+	+	–
***Bam*H I**[b]	–	++	++	++
Ban **I**	++	++	+	+
Ban **II**	++	++	++	++
Bbu **I**	++	++	–	–
Bbv I	++	++	++	++
Bcl **I**	–	++	++	–
Bgl **I**	–	++	++	++
Bgl **II**	+	++	++	++
***Bss*H II**	++	++	++	++
***Bsp*1286 I**	++	++	+	–
***Bst*71 I**	+	+	++	++
***Bst*E II**	–	+	++	++
*Bst*N I	+	+	++	++
***Bst*X I**	+	++	++	++
***Bst*Z I**	–	–	+	++
*Bsu*36 I	–	+	++	+
Cfo **I**	++	++	–	–
Cla **I**	++	++	++	++
Csp **I**	–	+	+	++
***Csp*45 I**	++	++	–	–
Dde **I**	+	++	++	++
Dpn **I**	–	++	++	++
Dra **I**	+	++	–	–
***Eco*47 III**	–	+	++	+
***Eco*R I**[b]	–	++	++	++
*Eco*R II	–	++	++	++
***Eco*R V**	–	–	–	++
*Fnu*4H I	++	++	+	–
*Fnu*D II	++	–	–	–
Fok I	++	++	++	++
Hae II	++	++	++	+
Hae **III**	++	++	++	++
Hga I	++	++	–	–
*Hgi*A I	–	–	+	++
Hha **I**	–	–	–	+
Hinc **II**	+	++	++	++
Hind **III**	+	++	++	+
Hinf **I**	+	++	++	++
Hpa **I**	–	++	++	–
Hpa **II**	++	++	–	–
Hph I	++	++	++	–
Kpn **I**	++	–	–	–
Mbo **I**	+	++	++	++
Mbo **II**	++	++	++	++
Mlu **I**	+	++	++	–
Mnl I	++	++	++	++
Msp **I**	++	++	++	++
Mst I	–	++	++	++
Mst II	–	+	++	++
Nae I	++	++	++	–
Nar **I**	++	++	+	+

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Table 4. NaCl Concentration Effects on Restriction Enzyme Activities.

	NaCl Concentration (mM)			
Enzyme	0	50	100	150
Nci I	++	++	+	–
***Nco* I**	–	+	++	++
Nde I	–	–	+	++
***Nhe* I**	++	++	++	++
***Not* I**	–	++	++	++
Nru I	–	++	++	++
***Nsi* I**	+	+	+	+
***Pst* I**	++	++	++	++
***Pvu* I**	–	+	++	++
***Pvu* II**	++	++	++	++
***Rsa* I**	++	++	++	++
***Sac* I**	++	+	+	–
***Sac* II**	++	++	–	–
***Sal* I**	–	–	+	++
***Sau*3A I**	++	++	++	++
***Sau*96 I**	++	++	++	++
***Sca* I**	–	++	++	+
*Scr*F I	+	++	++	++
*Sfa*N I	–	–	++	++
***Sfi* I**	++	++	++	++
***Sin* I**	++	–	–	–
***Sna*B I**	++	++	+	–
***Sma* I**[c]	+	+	+	+
***Spe* I**	–	++	++	++
***Sph* I**	–	–	++	++
***Spo* I**	–	++	++	+
***Ssp* I**	–	++	++	++
Sst I	++	++	–	–
***Stu* I**	++	++	++	++

	NaCl Concentration (mM)			
Enzyme	0	50	100	150
***Sty* I**	++	++	++	++
***Taq* I**[d]	++	++	++	–
Tha I	++	++	–	–
***Tth*111 I**	++	++	++	–
***Xba* I**	–	++	++	++
***Xho* I**	+	++	++	++
***Xho* II**	+	++	+	+
***Xma* I**	++	++	++	–
Xma III	++	++	++	–
Xmn I	++	++	–	–
Xor II	++	–	–	–

Key:

++ = indicates >50% activity.
+ = indicates 10–50% activity.
– = indicates >10% activity.

Notes:

The enzymes listed in boldface type are manufactured by Promega.

Reaction conditions used to assemble this table were 10mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 0.1mg/ml BSA at the optimum temperature for each enzyme.

a. Requires at least 50mM KCl for activity.

b. Star activity may be observed at low salt concentrations.

c. Requires at least 15mM KCl and an incubation temperature of 25–30°C for activity.

d. Incubations must be performed at 65–67°C.

Table 5. Restriction Enzymes Susceptible to Star Activity.

A restriction enzyme is said to exhibit star activity when it cleaves nucleic acid sequences that differ from its recognition sequence as a result of altered incubation conditions. Factors which may result in star activity include:

1. Non-optimal pH
2. Substitution of Co^{2+}, Mn^{2+}, or Zn^{2+} for Mg^{2+}
3. Increased enzyme concentration
4. Reduced salt concentration
5. Presence of organic solvents (>1%)
6. Presence of high glycerol

Enzymes	Conditions	Reference
Apy I	A,B	1
***Ava* I**	A,B	2
***Bam*H I**	A,B,C,D	2
***Bss*H II**	A,B	15
Bst I	A,B	2
***BstZ* I**	C	15
Bsv I	A,B	3
***Dde* I**	A,B	4
***Eco*R I**	A,B,C,D	2
***Eco*R V**	A	1
***Hha* I**	A,B	2
***Hin*d III**	C,D	5,13,14
***Hin*f I**	A,D	13
***Hpa* I**	A,B	2
***Kpn* I**	A,B	6
*Pae*R7 I	A,B,C	7
***Pst* I**	A,B	8
***Pvu* II**	A,B	9
***Sal* I**	A,B	10
*Sau*3A I	A,B	13
***Sca* I**	A,B,C,D	11,13
Sst I	A,B	2
Sst II	A,B	2
***Taq* I**	C	16
***Tth*111 I**	A	12
***Xba* I**	A,B	2

Note: The enzymes listed in boldface type are manufactured by Promega.

Key:

A - glycerol (12-20%)
B - enzyme:DNA ratio (25u/µg)
C - absence of NaCl
D - Mn^{2+} substituted for Mg^{2+}

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Restriction Enzymes and Linkers

Table 6. Isoschizomers of Commercially Available Restriction Enzymes.

Enzyme	Isoschizomer	Recognition Sequence
Aat II	*Stu* I	GACGT▼C
Acc I	—	GT▼(A/C)(G/T)AC
Acc III	*Bsp*M II	T▼CCGGA
Acy I	*Aha* II *Bbi* II *Hin*1 I	GPu▼CGPyC
Aha II	**Acy I**	GPu▼CGPyC
Aha III	**Dra I**	TTT▼AAA
Alu I	—	AG▼CT
Alw44 I	*Apa*L I	G▼TGCAC
Alw26 I	*Bsm*A I	GTCTC(1/5)
Aoc I	**Bsu36 I**	CC▼TNAGG
Apa I		GGGCC▼C
*Apa*L I	**Alw44 I**	G▼TGCAC
Ase I	**Vsp I**	AT▼TAAT
Asn I	**Vsp I**	AT▼TAAT
Asp I	**Tth111 I**	CAAPuCA(11/9)
*Asp*718 I	**Kpn I**	GGTAC▼C
Asu I	**Sau96 I** *Nsp* IV *Cfr*13 I	G▼GNCC
Asu II	**Csp45 I**	TT▼CGAA
Ava I	*Nsp* III	CPyCGPuG
Ava II	*Eco*47 I **Sin I**	G▼G(A/T)CC
Ava III	**Nsi I**	ATGCA▼T
Bal I	*Msc* I	TGG▼CCA
BamH I	*Bst* I	G▼GATCC
Ban II	*Hgi*J II	GPuGCPy▼C
Ban III	**Cla I**	AT▼CGAT
Bbe I	**Nar I**[3]	GGCGC▼C
Bbi II	**Acy I**	GPu▼CGPyC
Bbu I	**Sph** I	GCATG▼C
Bbv I	**Bst71 I**	GCAGC(8/12)
Bcl I	—	T▼GATCA
Bgl I	—	GCCNNNN▼NGGC
Bgl II	—	A▼GATCT
Bmy I	**Bsp1286 I**	G(G/A/T)GC(C/A/T)▼C
*Bse*P I	**BssH II**	G▼CGCGC
*Bsm*A I[2]	**Alw26 I**	GTCTC(1/5)
Bsp1286 I	*Sdu* I *Bmy* I	G(G/A/T)GC(C/A/T)▼C
*Bsp*M II	**Acc III**	T▼CCGGA
BssH II	*Bse*P I	G▼CGCGC
Bst I	**BamH I**	G▼GATCC
Bst71 I	*Bbv* I	GCAGC
*Bst*B I	**Csp45 I**	TT▼CGAA
BstE II	—	G▼GTNACC
BstX I	—	CCANNNNN▼NTGG
*Bst*Y I	**Xho II** *Mfl* I	Pu▼GATCPy
BstZ I	**Eco52 I** *Eag* I *Xma* III	C▼GGCCG
Bsu36 I	*Sau* I *Mst* II *Cvn* I *Aoc* I *Eco*81 I	CC▼TNAGG
Cfo I	**Hha I** *Hin*P1 I	GCG▼C
*Cfr*13 I	**Asu I** **Sau96 I**	G▼GNCC
Cla I	*Ban* III	AT▼CGAT
Csp I	*Rsr* II	CG▼G(A/T)CCG
Csp45 I	*Asu* II *Bst*B I *Nsp* V *Sfu* I	TT▼CGAA
Cvn I	**Bsu36 I**	CC▼TNAGG

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Enzyme	Isoschizomer	Recognition Sequence
***Dde* I**	—	C▼TNAG
***Dpn* I**[4]	—	G^{m6}ATC
***Dra* I**	*Aha* III	TTT▼AAA
Eag I	***Eco52* I** ***BstZ* I** *Xma* III	C▼GGCCG
***EcoR* I**		G▼AATTC
***EcoR* V**	—	G▼ATATC
*Eco*T14	***Sty* I**	C▼C(A/T)(A/T)GG
*Eco*T22 I	***Nsi* I**	ATGCA▼T
*Eco*47 I	***Ava* II** ***Sin* I**	G▼G(A/T)CC
***Eco47* III**	—	AGC▼GCT
***Eco52* I**	***BstZ* I** *Xma* III *Eag* I	C▼GGCCG
*Eco*81 I	***Bsu36* I**	CC▼TNAGG
*Eco*105 I	***SnaB* I**	TAC▼GTA
*Eco*130 I	***Sty* I**	C▼C(A/T)(A/T)GG
***Fok* I**[2]	—	GGATG(9/13)
***Hae* II**	—	PuGCGC▼Py
***Hae* III**	*Pal* I	GG▼CC
Hap II	***Hpa* II** ***Msp* I**	C▼CGG
*Hgi*J II	***Ban* II**	GPuGCPy▼C
***Hha* I**	***Cfo* I** *HinP*1 I	GCG▼C
*Hin*1 I	***Acy* I**	GPuCGPyC
***Hinc* II**	*Hind* II	GTPy▼PuAC
Hind II	***Hinc* II**	GTPy▼PuAC
***Hind* III**	—	A▼AGCTT
***Hinf* I**	—	G▼ANTC
*HinP*1 I	***Hha* I** ***Cfo* I**	GCG▼C
***Hpa* I**		GTT▼AAC
***Hpa* II**[5]	*Msp* I *Hap* II	C▼CGG

Enzyme	Isoschizomer	Recognition Sequence
***Kpn* I**	*Asp*718 I	GGTAC▼C
Ksp I	***Sac* II**	CCGC▼GG
***Mbo* I**	***Sau3A* I** *Nde* II	▼GATC
***Mbo* II**[2]	—	GAAGA(8/7)
Mfl I	***Xho* II**	Pu▼GATCPy
***Mlu* I**		A▼CGCGT
Mra I	***Sac* II**	CCGC▼GG
Msc I	***Bal* I**	TGG▼CCA
***Msp* I**[5]	***Hpa* II** *Hap* II	C▼CGG
Mst II	***Bsu36* I**	CC▼TNAGG
***Nar* I**[3]	*Bbe* I	GG▼CGCC
***Nco* I**	—	C▼CATGG
Nde II	***Mbo* I** ***Sau3A* I**	▼GATC
Nhe I		G▼CTAGC
***Not* I**		GC▼GGCCGC
Nru I	***Spo* I**	TCG▼CGA
***Nsi* I**	*Ava* III *Eco*T22 I	ATGCA▼T
Nsp III	***Ava* I**	C▼PyCGPuG
Nsp IV	***Asu* I** ***Sau96* I**	G▼GNCC
Nsp V	***Csp45* I**	T▼TCGAA
*Pae*R7 I	***Xba* I**	T▼CTAGA
Pal I	***Hae* III**	GG▼CC
***Pst* I**		CTGCA▼G
***Pvu* I**	*Xor* II	CGAT▼CG
***Pvu* II**		CAG▼CTG
***Rsa* I**		GT▼AC
Rsr II	***Csp* I**	CG▼G(A/T)CCG
***Sac* I**	*Sst* I	GAGCT▼C
***Sac* II**	*Sst* II *Ksp* I *Mra* I	CCGC▼GG

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Restriction Enzymes and Linkers

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Table 6. Isoschizomers of Commercially Available Restriction Enzymes.

Enzyme	Isoschizomer	Recognition Sequence
***Sal* I**		G▼TCGAC
Sau I	***Bsu*36 I**	C▼CTNAGG
***Sau*3A I**	***Mbo* I** *Nde* II	▼GATC
***Sau*96 I**	***Asu* I** *Cfr*13 I	G▼GNCC
***Sca* I**		AGT▼ACT
Sdu I	***Bsp*1286 I**	G(G/A/T)GC(C/A/T)▼C
Sfi I		GGCCNNNN▼NGGCC
Sfu I	***Csp*45 I**	
***Sin* I**	***Ava* II** *Eco*47 I	G▼G(A/T)CC
***Sma* I**	***Xma* I**	CCC▼GGG
***SnaB* I**	*Eco*105 I	TAC▼GTA
***Spe* I**		A▼CTAGT
***Sph* I**	***Bbu* I**	GCATG▼C
***Spo* I**	*Nru* I	TCG▼CGA
***Ssp* I**		AAT▼ATT
Sst I	***Sac* I**	GAGCT▼C
Sst II	***Sac* II**	CCGC▼GG
***Stu* I**	*Aat* I	AGG▼CCT
***Sty* I**	*Eco*130 I *Eco*T14 I	C▼C(A/T)(A/T)GG
***Taq* I**	*Tth*HB8 I	T▼CGA
***Tth*111 I**[2]	*Asp* I	CAAPuCA(11/9)
*Tth*HB8 I	***Taq* I**	T▼CGA
***Vsp* I**	*Ase* I *Asn* I	AT▼TAAT
***Xba* I**		T▼CTAGA
***Xho* I**	*Pae*R7 I	C▼TCGAG
***Xho* II**	*Bst*Y I *Mfl* I	Pu▼GATCPy

Enzyme	Isoschizomer	Recognition Sequence
***Xma* I**	***Sma* I**	CCC▼GGG
Xma III	***Eco*52 I** ***BstZ* I** *Eag* I	C▼GGCCG
Xor II	***Pvu* I**	CGAT▼CG

Key:

N = A, C, G or T
Pu = A or G
Py = C or T

Notes:

1. The enzymes listed in boldface type are manufactured by Promega
2. The locations of cleavage sites falling outside the recognition site are indicated in parentheses. For example, GTCTC(1/5) indicates cleavage at:
   5′...GTCTCN▼...3′
   3′...CAGAGNNNNN▲...5′
3. Note that, although *Nar* I and *Bbe* I share a recognition sequence, the cut sites differ.
4. *Dpn* I is unique among commercially available restriction enzymes in requiring methylation of a nucleotide (adenine) in its recognition sequence in order to cut. Therefore, *Dpn* I can not be substituted for other enzymes recognizing the GATC sequence (e.g., *Mbo* I and *Sau*3A I).
5. Although *Hpa* II and *Msp* I recognize the same nucleotide sequence, *Hpa* II is sensitive to methylation of either cytosine in its recognition sequence while *Msp* I is sensitive only to methylation of the external cytosine. These enzymes may not be interchanged for all applications.

Reference:

Roberts, R. J. (1990) Restriction enzymes and their isoschizomers. *Nucl. Acids Res.* **18**, 2331-2365.

Table 7. The Effect of Site-Specific Methylation on Promega Restriction Enzymes.

This table lists the sensitivities of several Promega restriction enzymes to site-specified methylations at ^{m6}A, ^{m5}C, ^{m4}C and ^{hm5}C. These four modifications are commonly found in DNA of bacteria, eukaryotes, and their viruses. Many strains of *E. coli* contain the site-specific *dam* and *dcm* DNA methylases. The *dam* methylase, encoded by the *dam* gene, methylates the N^{8} position of the adenine residue in the DNA sequence 5′...G^{m8}ATC...3′. The *dcm* gene encodes the *dcm* methylase which methylates the internal cytosine residues in DNA sequences 5′...CCTGG...3′ and 5′...CCAGG...3′. DNA isolated from strains expressing these methylases may be resistant to cleavage at some or all sites for the restriction enzymes listed below.

Restriction Enzyme	Recognition Sequence	Methylated Sites Cut	Methylated Sites Not Cut
Acc I[1]	GT(A/C)(G/T)AC	N/A	GT(A/C)(G/T)^{m6}AC
Acc III	TCCGGA	T^{m5}CCGGA	TCCGG^{m6}A
Alu I	AGCT	N/A	AG^{m5}CT AG^{m4}CT AG^{hm5}CT ^{m6}AGCT
Apa I	GGGCCC	N/A	GGG^{m5}CCC GGGCC^{m5}C
Asu II	TTCGAA	TT^{m5}CGAA	N/A
Ava I[2]	CPyCGPuG	C^{m5}CCGGG	^{m5}CPyCGPuG CPy^{m5}CGPuG CTCG^{m6}AG
Ava II	GG(A/T)CC	N/A	GG(A/T)C^{m5}C GG(A/T)^{m5}CC GG(A/T)^{hm5}C^{hm5}C
Bal I[3]	TGGCCA	N/A	TGG^{m5}CCA TGGC^{m5}CA
*Bam*H I	GGATCC	GGATC^{m5}C GG^{m6}ATCC GG^{m6}ATC^{m5}C	GGATC^{4m}C GGAT^{m5}CC GGAT^{hm5}C^{hm5}C
Ban II	GPuGCPyC	N/A	GPuG^{m5}CPyC
Bcl I	TGATCA	TGAT^{m5}CA	TG^{m6}ATCA TGA^{hm5}CA
Bgl I[4]	GCCNGGC	GC^{m5}CNGGC	GCCNGG^{m5}C
Bgl II	AGATCT	AG^{m6}ATCT	AGAT^{m5}CT AGAT^{hm5}CT
*Bst*E II[5]	GGTNACC	GGTNA^{m5}C^{m5}C	GGTNA^{hm5}C^{hm5}C
*Bst*XI	CCANTGG	N/A	^{m5}CCANTGG CC^{m6}ANTGG
Cfo I	GCGC	N/A	G^{hm5}CG^{hm5}C G^{m5}CGC
Cla I	ATCGAT	N/A	ATCG^{m6}AT AT^{m5}CGAT AT^{m5}CGAT
Csp I	CGG(A/T)CCG	N/A	CGG(A/T)^{m5}CCG ^{m5}CGG(A/T)C^{m5}CG
Dde I	CTNAG	N/A	^{m5}CTNAG ^{hm5}CTNAG
Dpn I[6]	G^{m6}ATC	G^{m6}ATC G^{m6}AT^{m5}C	GATC GAT^{m5}C
*Eco*R I[7]	GAATTC	GAATT^{hm5}C	GA^{m6}ATTC G^{m6}AATTC GAATT^{m5}C
*Eco*R V[8]	GATATC	GATAT^{m5}C	G^{m6}ATATC
Fok I[9]	CATCC	CATC^{m5}C CAT^{m5}CC	GG^{m6}ATG
Hae II[10]	PuGCGCPy	N/A	PuG^{m5}CGCPy PuG^{hm5}CG^{hm5}CPy
Hae III[11]	GGCC	GGC^{m5}C	GG^{m5}CC GG^{hm5}C^{hm5}C
Hha I	GCGC	N/A	G^{m5}CGC GCG^{m5}C G^{hm5}CG^{hm5}C
Hinc II	GTPyPuAC	GTPyPuA^{m5}C	GTPyPu^{m6}AC GTPyPuA^{hm5}C
Hind III	AAGCTT	N/A	^{m6}AAGCTT AAG^{m5}CTT AAG^{m5}CTT
Hinf I[12]	GANTC	GANT^{m5}C	G^{m6}ANTC GANT^{hm5}C
Hpa I	GTTAAC	GTTAA^{m5}C	GTTA^{m6}AC GTTAA^{hm5}C
Hpa II[13]	CCGG	N/A	C^{m5}CGG ^{m5}CCGG ^{m4}CCGG C^{m4}CGG ^{hm5}C^{hm5}CGG

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Table 7. The Effect of Site-Specific Methylation on Promega Restriction Enzymes.

Restriction Enzyme	Recognition Sequence	Methylated Sites Cut	Methylated Sites Not Cut
Kpn I[14]	GGTACC	GGTA^{m5}CC GGTAC^{m5}C GGTA^{m5}C^{m5}C	N/A
Mbo I[15]	GATC	GAT^{m5}C	G^{m6}ATC GAT^{hm5}C
Mbo II[16]	GAAGA	T^{m5}CTT^{m5}C	GAAG^{m6}A
Mlu I	ACGGCGT	^{m6}ACGCGT	A^{m5}CGCGT
Msp I[17]	CCGG	C^{m5}CGG ^{m4}CCGG C^{m4}CGG	^{m5}CCGG ^{hm5}C^{hm5}CGG
Nco I	CCATGG	N/A	^{m5}CCATGG
Nhe I	GCTAGC	N/A	GCTAG^{m5}C
Not I	GCGGCCGC	GCGGCCG^{m5}C	GCGG^{m5}CCGC GCGGC^{m5}CGC
Pst I	CTGCAG	N/A	CTGC^{m6}AG ^{m5}CTGCAG
Pvu I	CGATCG	CG^{m6}ATCG	CGAT^{m4}CG CGAT^{m5}CG
Pvu II	CAGCTG	N/A	CAG^{m4}CTG CAG^{m5}CTG
Rsa I[18]	GTAC	GTA^{m5}C	N/A
Sac I	GAGCTC	G^{m6}AGCTC	GAG^{m5}CTC
Sac II	CCGCGG	N/A	^{m5}CCGCGG
Sal I	GTCGAC	N/A	GTCG^{m6}AC GT^{m5}CGAC
*Sau*3A I[19]	GATC	G^{m6}ATC	GAT^{m5}C GAT^{m4}C GAT^{hm5}C
*Sau*96 I	GGNCC	N/A	GGN^{m5}CC GGNC^{m5}C GGN^{hm5}C^{hm5}
ScrF I	CCNGG	^{m5}CCNGG	C^{m5}CNGG
Sfi I	GGCCNGGCC	GG^{m5}CCNGG^{m5}CC GGCCNGGC^{m5}C	N/A
Sin I	GG(A/T)CC	N/A	GG(A/T)^{5m}CC
Sma I[20]	CCCGGG	C^{m5}CCGGG	^{m5}CCCGGG CC^{m5}CGGG ^{m4}CCCGGG C^{m4}CCGGG CC^{m4}CGGG
Spe I	ACTAGT	N/A	^{m6}ACTAGT
Sph I	GCATGC	GCATG^{m5}C G^{hm5}GATG^{hm5}C	N/A
Stu I	AGGCCT	N/A	AGG^{m5}CCT AGGC^{m5}CT
Taq I[21]	TCGA	T^{m5}CGA T^{hm5}CGA	TCG^{m6}A
Xba I	TCTAGA	N/A	T^{m5}CTAGA TCTAG^{m6}A T^{hm5}CTAGA
Xho I	CTCGAG	N/A	CT^{m5}CGAG CTCG^{m6}AG ^{m5}CTCGAG
Xma I[22]	CCCGGG	CC^{m5}CGGG	^{m4}CCCGGG C^{m4}CCGGG CC^{m4}CGGG ^{m5}CCCGGG

Key:

^{m6}A = 6-methyladenine (*dam* methylation)
^{m5}C = 5-methylcytosine (*dcm* methylation)
^{m4}C = 4-methylcytosine
^{hm5}C = 5-hydroxymethylcytosine
N = A, C, G or T
Pu = A or G
Py = C or T

Notes:

1. *Acc* I nicking occurs slowly in the unmethylated strand of the hemi-methylated sequence GT(A/C)(G/T)A^{m5}C.
2. *Ava* I nicking occurs slowly in the unmethylated strand of the hemi-methylated sequence CTCG^{m6}AG/CTCGAG.
3. *Bal* I sites overlapping *dcm* sites (TGGC^{m5}CAGG) are cut 50-fold slower than unmethylated sites.

(continued on next page)

Restriction Enzymes and Linkers

4. *Bgl* I cleavage at certain hemi-methylated ^{m5}C sites varies (overlapping M • *Msp* I - *Bgl* I and M • *Hpa* II - *Bgl* I sites). ^{m5}C bi-methylated M • *Hae* III - *Bgl* I sites are completely refractory to *Bgl* I.
5. *Bst*E II cuts the fully ^{m5}C substituted phage XP12 DNA.
6. *Dpn* I cleavage requires adenine methylation on both DNA strands. Isoschizomers of *Dpn* I include *Cfu* I, *Nan* II, *Nmu*E I, *Nmu*D I and *Nsu*D I.
7. *Eco*R I can not cut hemi-methylated G^{m6}AATTC/GAATTC sites. Bi-methylated GA^{m6}ATTC/GA^{m6}ATTC sites are not cut by *Eco*R I or *Rsr* I. *Eco*R I shows a reduced rate of cleavage at hemi-methylated GAATT^{m5}C and does not cut an oligonucleotide that contains GAATT^{m5}C in both strands.
8. *Eco*R V cuts the fully ^{m5}C substituted phage XP12 DNA.
9. *Fok* I cuts about 2 to 4-fold more slowly at CATC^{m5}C than at unmodified sites.
10. *Hae* II gives various rate effects when its recognition sequence is ^{m5}C methylated at different positions. M • *Hae* II may be ^{m5}C specific.
11. *Hae* III nicking occurs in the unmethylated strand of the hemi-methylated sequence GG^{m5}CC/GGCC. M • *Hga* I may be ^{m4}C or ^{m6}A specific.
12. *Hin*f I cuts GANT^{m5}C. However, detectable rate differences are observed between unmethylated, hemi-methylated (GANT^{m5}C/GANTC) and bi-methylated (GANT^{m5}C/GANT^{m5}C) target sequences. *Hin*f I does cut phage XP12 DNA, although at a reduced rate. *Hin*f I cuts unmethylated GANTC faster than hemi-methylated GANT^{m5}C/GANTC, which is cut faster than GANT^{m5}C/GANT^{m5}C. However, the rate difference between unmethylated and fully methylated *Hin*f I sites is only about 10-fold.
13. *Hpa* II nicking occurs in the unmethylated strand of the hemi-methylated sequence ^{m5}CCGG/CCGG.
14. *Kpn* I sensitivity to hemi-methylated GGTA^{m5}CC and GGTAC^{m5}C sites is in dispute. It is possible that rate effects are observed at certain ^{m5}C methylated *Kpn* I sites, especially at low enzyme-to-substrate ratios. *Kpn* I cuts the fully ^{m5}C substituted phage XP12 DNA.
15. *Mbo* I isoschizomers that are sensitive to G^{m6}ATC include *Bss*G II, *Bsa*P I, *Bst*X II, *Bst*E III, *Cpa* I, *Dpn* II, *Fnu*A II, *Fnu*C I, *Mme* II, *Mno* III, *Mos* I, *Nde* II, *Nfl* I, *Nla* II, *Nsu* I, *Sin*M I.
16. *Mbo* II cuts the fully ^{m5}C substituted phage XP12 DNA although certain hemi-methylated ^{m5}C-containing substrates are reported not to be cut.
17. *Msp* I cuts the unmethylated strand and methylated strand of C^{m5}CGG/CCGG and C^{m4}CGG/CCGG duplexes. *Msp* I cuts very slowly at GGC^{m5}CGG. An M • *Msp* I clone methylates ^{m5}CCGG. However, there is a report that *Moraxella* sp. chromosomal DNA is methylated at ^{m5}C^{m5}CGG.
18. *Rsa* I cuts the fully ^{m5}C substituted phage XP12 DNA. M • *Rsa* I may be ^{m4}C or ^{m6}A specific.
19. *Sau*3A I nicking occurs in the unmethylated strand of the hemi-methylated sequence GAT^{5m}C/GATC. *Sau*3A I cuts at a reduced rate at ^{m6}AGATC. *Sau*3A I isoschizomers that are insensitive to G^{m6}ATC include *Bce*243 I, *Bsp*67 I, *Bsp*A I,*Bsp*P II, *Bsr*P II, *Cpe* I, *Fnu*E I, *Mth* I, *Nsi*A I, *Pfa* I.
20. *Sma* I nicking occurs in the unmethylated strand of the hemi-methylated strand of the hemi-methylated sequence CC^{m5}CGGG/CCCGGG. M • *Sma* I may be ^{m5}C specific. *Sma* I may cut C^{m5} C^{m5}CGGG methylated DNA. Possibly the second methylation negates the effect of CC^{m5}CGGG. There are conflicting results regarding *Sma* I: ^{m5}CCCGGG is not cut when modified by M • *Aqu* I methyltransferase or at overlapping M • *Hae* III - *Sma* I sites (GG^{m5}CCCGGG); other investigators have reported that *Sma* I cuts at a reduced rate at hemi-methylated ^{m5}CCCGGG sites.
21. *Taq* I cuts very slowly at T^{hm5}CGA. *Taq* I cuts the fully ^{m5}C substituted phage XP12 DNA.
22. *Xma* I is claimed not to cut CC^{m5}CGGG in one report.

References:

1. McClelland, M. and Nelson, M. (1988) *Gene* **74**, 291-304.
2. Nelson, M. and McClelland, M. (1988) *Promega Notes* **15**.

Restriction Enzymes and Linkers

Table 8. Isoschizomer Enzyme Pairs that Differ in their Ability to Cut Methylated DNA.

Methylated Sequence	Not Cut By	Cut By
C^{m5}CGG	***Hpa* II**	***Msp* I**
CC^{m5}CGGG	***Sma* I**	***Xma* I**
C^{m5}CPuGG	*Eco*R II	*Bst*N I
TCCGG^{m6}A	***Acc* III**	*Bsp*M II
G^{m6}ATC	***Mbo* I**	***Sau3A* I** ***Dpn* I**
PuG^{m6}ATCPy	*Mfl* I	*Xho* II

Key:

Pu = A or G
Py = C or T

Note: The enzymes listed in boldface type are manufactured by Promega.

Reference:

1. Nelson, M. and McClelland, M. (1987) *Nucl. Acids Res.* **15**, Suppl.:r219.

Table 9. Frequency of Restriction Enzyme Cutting in Common DNA Substrates.

		Frequency of Cutting				
Enzyme	Recognition Site	λ	Ad2	SV40	ϕX174	pBR322
***Aat* II**	GACGT▼C	10	3	0	1	1
***Acc* I**	GT▼(A/C)(G/T)AC	9	17	1	2	2
***Acc* III**	T▼CCGGA	24	8	0	0	1
***Acy* I**	GPu▼CGPyC	40	44	0	7	6
Aha II	GPu▼CGPyC	40	44	0	7	6
Aha III	TTT▼AAA	13	12	12	2	3
***Alu* I**	AG▼CT	143	158	34	24	16
***Alw26* I**	GTCTC(1/5)	37	60	2	4	3
***Alw44* I**	G▼TGCAC	4	7	0	1	3
***Apa* I**	GGGCC▼C	1	12	1	0	0
***Asu* I**	G▼GNCC	74	164	11	2	15
Asu II	TT▼CGAA	7	1	0	0	0
***Ava* I**	C▼PyCGPuG	8	40	0	1	1
***Ava* II**	G▼G(A/T)CC	35	73	6	1	8
***Bal* I**	TGG▼CCA	18	17	0	0	1
***Bam*H I**	G▼GATCC	5	3	1	0	1

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Restriction Enzymes and Linkers

Enzyme	Recognition Site	Frequency of Cutting λ	Ad2	SV40	φX174	pBR322
Ban I	G▼GPyPuCC	25	57	1	3	9
***Ban* II**	GPuGCPy▼C	7	50	2	0	2
***Bbu* I**	GCATC▼C	6	8	2	0	1
Bbv I[2]	GCAGC(8/12)	199	179	22	14	21
***Bcl* I**	T▼GATCA	8	5	1	0	0
***Bgl* I**	GCCNNNN▼NGGC	29	20	1	0	3
***Bgl* II**	A▼GATCT	6	11	0	0	0
Bsm I	GAATGC	46	10	4	3	1
***Bsp*1286 I**	G(A/G/T)GC(A/C/T)▼C	38	105	4	3	10
***Bss*H II**	G▼CGCGC	6	52	0	1	0
Bst I	G▼GATCC	5	3	1	0	1
***Bst*71 I**	GCAGC	199	179	22	14	21
***Bst*E II**	G▼GTNACC	13	10	0	0	0
*Bst*N I	CC▼(A/T)GG	71	136	17	2	6
***Bst*X I**	CCANNNNN▼NTGG	13	10	1	3	0
***Bst*Z I**	CGGCCG	2	19	0	0	1
***Bsu*36 I**	CC▼TNAGG	2	0	0	0	0
***Cfo* I**	GCG▼C	215	375	2	18	31
***Cla* I**	AT▼CGAT	15	2	0	0	1
***Csp* I**	CG▼G(A/T)CGG	5	2	0	0	0
***Csp*45 I**	TT▼CGAA	7	1	0	0	0
***Dde* I**	C▼TNAG	104	97	20	14	8
***Dpn* I**	G^{m6}A▼TC	116	0	0	0	22
***Dra* I**	TTT▼AAA	13	12	12	2	3
***Eco*52 I**	C▼GGCCG	2	19	0	0	1
***Eco*R I**	G▼AATTC	5	5	1	0	1
***Eco*R V**	GAT▼ATC	21	9	1	0	1
***Eco*47 III**	AGC▼GCT	2	13	1	0	4
*Fnu*D III	GCG▼C	215	375	0	18	31
*Fnu*4H I	GC▼NGC	380	411	24	31	42
***Fok* I**[2]	GGATG(9/13)	150	78	11	8	12
Fsp I	TGC▼GCA	15	17	0	1	4
***Hae* II**	PuGCGC▼Py	48	76	1	8	11
***Hae* III**	GG▼CC	149	216	18	11	22
Hga I	GACGC(5/10)	102	87	0	14	11

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Restriction Enzymes and Linkers

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Table 9. Frequency of Restriction Enzyme Cutting in Common DNA Substrates.

		Frequency of Cutting				
Enzyme	Recognition Site	λ	Ad2	SV40	φX174	pBR322
HgiA I	G(A/T)GC(A/T)▼C	28	38	0	3	8
***Hha* I**	GCG▼C	215	375	2	18	31
***Hinc* II**	GTPy▼PuAC	35	25	7	13	2
***Hind* III**	A▼AGCTT	7	12	6	0	1
***Hinf* I**	G▼ANTC	148	72	10	21	10
HinP1 I	G▼CGC	215	2	2	18	31
***Hpa* I**	GTT▼AAC	14	6	4	3	0
***Hpa* II**	C▼CGG	328	171	1	5	26
Hph I[2]	GGTGA(8/7)	168	99	4	9	12
***Kpn* I**	GGTAC▼C	2	8	1	0	0
***Mbo* I**	▼GATC	116	87	8	0	22
***Mbo* II**[2]	GAAGA(8/7)	130	113	16	11	11
***Mlu* I**	A▼CGCGT	7	5	0	2	0
Mnl I	CCTC(7/7)	262	397	51	34	26
***Msp* I**	C▼CGG	328	171	1	5	26
Mst II	CC▼TNAGG	2	7	0	0	0
Nae I	GCC▼GGC	1	13	1	0	4
***Nar* I**	GG▼CGCC	1	20	0	2	4
Nci I	CC▼(G/C)GG	114	97	0	1	10
***Nco* I**	C▼CATGG	4	20	3	0	0
Nde I	CA▼TATG	7	2	2	0	1
***Nhe* I**	G▼CTAGC	1	4	0	0	1
Nla III	CATG▼	181	183	17	22	26
Nla IV	GGN▼NCC	82	178	16	6	24
***Not* I**	GC▼GGCCGC	0	7	0	0	0
Nru I	TCG▼CGA	5	5	0	2	1
***Nsi* I**	ATGCA▼T	14	9	3	0	0
PaeR7 I	C▼TCGAG	1	6	0	1	0
***Pst* I**	CTGCA▼G	28	30	2	1	1
***Pvu* I**	CGAT▼CG	3	7	0	0	1
***Pvu* II**	CAG▼CTG	15	24	3	0	1
***Rsa* I**	GT▼AC	113	83	12	11	13
Rsr II	CG▼G(A/T)CGG	5	2	0	0	0

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Restriction Enzymes and Linkers

Enzyme	Recognition Site	Frequency of Cutting λ	Ad2	SV40	ϕX174	pBR322
Sac I	GAGCT▼C	2	16	0	0	0
Sac II	CCGC▼GG	4	33	0	1	0
Sal I	G▼TCGAC	2	3	0	0	1
_Sau_3A I	▼GATC	116	87	8	0	22
_Sau_96 I	G▼GNCC	74	164	11	2	15
Sca I	AGT▼ACT	5	5	0	0	1
_Scr_F I	CC▼NGG	185	233	17	3	16
_Sfa_N I[2]	GCATC(5/9)	169	84	6	12	22
Sfi I	GGCCNNNN▼NGGCC	0	3	1	0	0
Sin I	GG(A/T)CC	35	73	6	1	8
Sma I	CCC▼GGG	3	12	0	0	0
_Sna_B I	TAC▼GTA	1	0	0	0	0
Spe I	A▼CTAGT	0	3	0	0	0
Sph I	GCATG▼C	6	8	2	0	1
Spo I	TCG▼CGA	5	5	0	2	1
Ssp I	AAT▼ATT	20	5	1	6	1
Stu I	AGG▼CCT	6	11	7	1	0
Sty I	C▼C(A/T)(A/T)GG	10	44	8	0	1
Taq I	T▼CGA	121	50	1	10	7
_Tth_111 I	GACN▼NNGTC	2	12	0	0	1
Vsp I	AT▼TAAT	17	3	3	2	3
Xba I	T▼CTAGA	1	5	0	0	0
Xho I	C▼TCGAG	1	6	0	1	0
Xho II	Pu▼GATCPy	21	22	3	0	8
Xma I	C▼CCGGG	3	12	0	0	0
Xma III	C▼GGCCG	2	19	0	0	1
Xmn I	GAANN▼NNTTC	24	5	0	3	2

Notes:

1. The enzymes listed in boldface type are manufactured by Promega.
2. The locations of cleavage sites falling outside the recognition site are indicated in parentheses. For example, GTCTC (1/5) indicates cleavage at:
   5′...GTCTCN▼...3′
   3′...CAGAGNNNN▲5′

Key:

N = A, C, G or T
Pu = A or G
Py = C or T

Restriction Enzymes and Linkers

Table 10. Comparison of Units of Restriction Enzyme Required to Digest Common DNA Substrates.

Enzyme	Lambda	pBR322	φX174RF	Enzyme	Lambda	pBR322	φX174RF
Aat II	1	1	–	*Eco*R I	1	5	–
Acc I	1	–	–	*Eco*R V	1	2	–
Acc III	–	–	–	*Fok* I	1	2	1
Acy I	1	–	–	*Hae* II	1	1-2	1
*Alw*26 I	1	2-3	2	*Hae* III	1	5	2
*Alw*44 I	1	2-3	1	*Hha* I	1	4	1
Alu I	1	2	1	*Hinc* II	1	5	2
Apa I	1	–	–	*Hind* III	1	2	–
Asu I	–	–	–	*Hinf* I	1	1	1
Ava I	1	–	–	*Hpa* I	1	–	1
Ava II	1	–	–	*Hpa* II	1	2	1
Bal I	1	2-3	1	*Kpn* I	1	–	–
*Bam*H I	1	3	–	*Mbo* I	1	2	–
Ban I	1	–	–	*Mbo* II	1	2	1
Ban II	1	–	–	*Mlu* I	1	–	–
Bbu I	1	–	–	*Msp* I	1	–	–
Bcl I	1	–	–	*Nar* I	1	5-10	–
Bgl I	1	–	–	*Nco* I	1	–	–
Bgl II	1	–	–	*Nhe* I	1	–	–
*Bsp*1286 I	1	2	1	*Not* I	–	–	–
*Bss*H II	1	–	1	*Nsi* I	1	–	–
*Bst*E II	1	–	–	*Pst* I	1	5	1
*Bst*X I	1	–	–	*Pvu* I	1	1-2	–
*Bsu*36 I	1	–	–	*Pvu* II	1	5	–
Cfo I	1	2-3	2	*Rsa* I	1	–	–
Cla I	1	–	–	*Sac* I	1	–	–
Csp I	1	–	–	*Sac* II	1	–	1
*Csp*45 I	1	–	–	*Sal* I	1	10-20	–
Dde I	1	2	1	*Sau*3A I	1	2-3	–
Dpn I	1	1	–	*Sau*96 I	1	–	–
Dra I	1	2-3	1	*Sca* I	1	5	–
*Eco*47 III	1	2	–	*Sfi* I	–	–	–
*Eco*52 I	1	2-3	–	*Sin* I	1	–	–

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Restriction Enzymes and Linkers

Enzyme	Lambda	pBR322	ϕX174RF
Sma I	1	–	–
*Sna*B I	1	–	–
Sph I	1	2-3	–
Spe I	–	–	–
Spo I	1	–	–
Ssp I	1	–	–
Stu I	1	–	–
Sty I	1	–	–
Taq I	1	10-15	–
*Tth*111 I	1	10	–
Vsp I	1	2-3	1
Xba I	1	–	–
Xho I	1	–	1
Xma I	1	–	–

Key: (–) The enzyme does not cut this DNA.

Note: All restriction enzymes listed are manufactured by Promega.

Table 11. Alphabetized List of Recognition Sequences for Enzymes Available from Promega.

A▼AGCTT	*Hind* III
AAT▼ATT	*Ssp* I
A▼CGCGT	*Mlu* I
A▼CTAGT	*Spe* I
A▼GATCT	*Bgl* II
AG▼CT	*Alu* I
AGG▼CCT	*Stu* I
AGT▼ACT	*Sca* I
AT▼CGAT	*Cla* I
ATGCA▼T	*Nsi* I
AT▼TAAT	*Vsp* I
CAG▼CTG	*Pvu* II
GGATG(9/13)	*Fok* I
CCAN5▼NTGG	*Bst*X I
C▼CATGG	*Nco* I
C▼CCGGG	*Xma* I
CCC▼GGG	*Sma* I
CCGC▼GG	*Sac* II
C▼CGG	*Hpa* II
C▼CGG	*Msp* I
CC▼TNAGG	*Bsu*36 I
C▼C(A/T)(A/T)GG	*Sty* I
CGAT▼CG	*Pvu* I
C▼GGCCG	*Eco*52 I, *BstZ* I
CG▼G(A/T)CCG	*Csp* I
C▼TCGAG	*Xho* I
C▼TCGAG	*Xba* I
CTGCA▼G	*Pst* I
C▼TNAG	*Dde* I
C▼PyCGPuG	*Ava* I
GAAGA(8/7)	*Mbo* II
G▼AATTC	*Eco*R I
GACGT▼C	*Aat* II
GACN▼NNGTC	*Tth*111 I
GAGAC	*Alw*26 I
GAGCT▼C	*Sac* I
G▼ANTC	*Hinf* I

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Table 11. Alphabetized List of Recognition Sequences for Enzymes Available from Promega.

GAT▼ATC	*Eco*R V
▼GATC	*Mbo* I
▼GATC	*Sau*3A I
G(mA)▼TC	*Dpn* I
GCAGC	*Bst*71 I
GCATG▼C	*Sph* I
GCCN4▼NGGC	*Bgl* I
GCG▼C	*Hha* I
G▼CGCGC	*Bss*H II
GC▼GGCCGC	*Not* I
G▼CTAGC	*Nhe* I
G(A/G/T)GC(A/C/T)▼C	*Bsp*1286 I
G▼GATCC	*Bam*H I
GGATG(9/13)	*Fok* I
GG▼CC	*Hae* III
GGCCN4▼NGGCC	*Sfi* I
GG▼CGCC	*Nar* I
GGGCC▼C	*Apa* I
G▼GNCC	*Sau*96 I
GGTAC▼C	*Kpn* I
G▼GTNACC	*Bst*E II
G▼G(A/T)CC	*Ava* II
GPu▼CGPyC	*Aha* II
GPuGCPy▼C	*Ban* II
GT▼AC	*Rsa* I
G▼TCGAC	*Sal* I
GTCTC(1/5)	*Alw*26 I
G▼TGCAC	*Alw*44 I
GT▼(A/C)(G/T)AC	*Acc* I
GT▼TAAC	*Hpa* I
GTPy▼PuAC	*Hinc* II
(A/C/T)AT▼CGAT(A/G/T)	*dam* + *Cla* I
PuGATCPy	*Xho* II
PuGCGC▼Py	*Hae* II
TAC▼GTA	*Sna*B I
TCACC	*Hph* I
T▼CCGGA	*Acc* III
T▼CGA	*Taq* I
TCG▼CGA	*Spo* I
T▼CTAGA	*Xba* I
TCTTC	*Mbo* II
T▼GATCA	*Bcl* I
TGG▼CCA	*Bal* I
TT▼CGAA	*Csp*45 I
TTT▼AAA	*Dra* I

Key:

N = A, C, G or T
Pu = A or G
Py = C or T

Note:

The locations of cleavage sites falling outside of the recognition site are indicated in parenthesis. For example, GTCTC(1/5) indicates cleavage at:
5′...GTCTCN▼...3′
3′...CAGAGNNNNN▲...5′

Restriction Enzymes and Linkers

Restriction Analysis of Megabase DNA Molecules.

The development of pulsed-field gel electrophoresis (1) has made possible the direct separation of chromosomal DNAs from a variety of organisms. This technology, in combination with infrequent-cutting restriction enzymes, has been used for the physical mapping of large regions of mammalian chromosomes (2).

Chromosomal DNA must be prepared in agarose blocks to minimize shear damage. Therefore, restriction enzymes used in the analysis of megabase DNA fragments must be able to function within agarose plugs, an environment clearly different from that of digestion reactions in solution (3). Higher ratios of enzyme units to DNA and longer incubation times are frequently needed, for example, to guarantee complete digestion.

Promega's genome qualified restriction enzymes have been qualified for digestions in agarose plugs. In addition, chromosomal DNA preparation, digestion, and gel separation parameters have been optimized for each genome qualified enzyme to ensure clean digestion products free of degraded background as well as sharp, clean banding patterns, accurately sized and easily visualized in ethidium bromide-stained gels. Optimized conditions for chromosomal DNA digestion are given below and in Table 12.

Optimized Protocol for Chromosomal DNA Digestion

1. Soak each agarose plug containing a chromosomal DNA sample in TE buffer for 30 minutes on ice.
2. Equilibrate each plug in 100µl of the appropriate enzyme buffer for 30 minutes on ice.
3. Add restriction enzyme and 2µl of 1mg/ml acetylated BSA to each tube and leave the reaction on ice for another 30 minutes.
4. Carry out the restriction digestion using the time and temperature specified in Table 12.
5. Terminate the reaction by adding 12µl of 0.5M EDTA.
6. Store the sample at 4°C for up to several days until use.

References:

1. Schwartz, D.C., *et al.* (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 18.
2. Smith, C.L., *et al.* (1987) *Science* **236**, 1448.
3. Hung, L.C., unpublished observations, Promega Corporation.

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Table 12. Parameters for Digestion of Chromosomal DNA by Promega's Genome Qualified Restriction Enzymes

Promega Enzymes	Recognition Sequence	Source	Genome Size (Mb)	Genome G+C (%)	Conditions for Digestion: Enzyme (u): DNA (µg)	Conditions for Digestion: T°C	Conditions for Digestion: Time (hr)	Number of Fragments
Bgl I*	GCCNNNN▼NGGC	*S. aureus*	3.0	34	30:2	37	3	20-25
*Bss*H I	G▼CGCGC	*S. aureus*	3.0	34	14:2	50**	4	many
*Eco*47 III	AGC▼GCT	*S. aureus*	3.0	34	8:2	37	4	many
Mlu I	A▼CGCGT	*S. aureus*	3.0	34	10:2	37	3	25-30
Sma I*	CCC▼GGG	*S. aureus*	3.0	34	20:2	22	3	18
Spo I*	TCG▼CGA	*S. aureus*	3.0	34	10:2	37	4	25-30
Cla I	AT▼CGAT	*M. bovis*	2.9	45	12:1	37	4	many
Csp I	CGG▼(A/T)CCG***	*M. bovis*	3.0	45	8:1	30	5	8
Nhe I	G▼CTAGC	*M. bovis*	2.9	45	5:1	37	3	20-25
Not I*	GC▼GGCCGC	*M. bovis*	2.9	45	5:1	37	3	7
Sal I*	G▼TCGAC	*M. bovis*	2.9	45	16:1	37	4	≈ 15
Spe I	A▼CTAGT	*M. bovis*	2.9	45	5:1	37	3	20-25
Ssp I*	AAT▼ATT	*M. bovis*	2.9	45	2:1	37	4	≈ 10
Xba I*	T▼CTAGA	*M. bovis*	2.9	45	10:1	37	3	25-30
Xho I*	C▼TCGAG	*M. bovis*	2.9	45	16:1	37	4	15-20
Bcl I*	T▼GATCA	*N. crassa*	45	—	30:2	50**	15	many
Sfi I*	GGCCNNNN▼NGGCC	*S. pombe*	14	45	5:1	50**	16	≈ 15

* These enzymes are available at high concentration: 40-80u/ml.
** Perform 50°C digestions under mineral oil.
*** Please inquire for the cut site location.

Table 13. Enzymes Used to Infrequently Cleave Mammalian Genomes.

The sizes of restriction fragments from the human cystic fibrosis (CF) and major histocompatibility complex (MHC) loci may be used as predictors of restriction fragment sizes in other regions of the human genome. In these examples, human genomic DNA was digested with the rare-cutting endonucleases indicated and individual restriction fragments were detected using probes to the CF and MHC loci. The values shown below are average restriction fragment sizes (ARFS) obtained with each endonuclease.

Average restriction fragment sizes may also be predicted statistically based on di- and trinucleotide frequencies in sequenced DNA (1), but these predictions can not take into account DNA sequences in intergenic regions nor the extensive methylation of mammalian DNA at CG pairs. CG methylation prevents cleavage at many sites, thus generating larger fragment sizes than would be predicted statistically (2).

Enzymes	Recognition Sequence	ARFS in Human CF Locus (3,4) (kb)	ARFS in Human MHC Locus (5,6,7,8) (kb)
***Not* I**[2]	GCGGCCGC	1,300	800
***Spo* I**[2]	TCGCGA	1,200	650
***Mlu* I**[2]	ACGCGT	400	400
***Csp* I**[2]	CGG(A/T)CCG	N/D	600
***Bss*H II**[2]	GCGCGC	400	300
***Sal* I**[2]	CTCGAC	400	300
***Sfi* I**[2]	GGCCN$_5$GGCC	400	200
***Sac* II**	CGGCGG	300	300
***Pvu* I**	CGATCG	N/D	450
***Xho* I**[2]	CTCGAG	400	200
Nae I	GCCGGC	350	400
***Cla* I**[2]	ATCGAT	N/D	200
***Eco52* I**[2]	CGGCCG	N/D	250
***Nar* I**	GGCGCC	N/D	200

Notes:

1. The enzymes listed in boldface type are manufactured by Promega.
2. Promega genome qualified enzyme.

References:

1. McClelland, M. and Nelson, M. (1987) *Gene Amplification and Analysis* **5**, 257-282.
2. McClelland, M. and Nelson, M. (1987) *Nucl. Acids Res.* **15**, r219-r230.
3. Poustka, A., *et al.* (1988) *Genomics* **2**, 337-345.
4. Drumm, M., *et al.* (1988) *Genomics* **2**, 346-354.
5. Ragoussis, J., *et al.* (1989) *Genomics* **4**, 301-308.
6. Lawrance, S., *et al.* (1989) *Science* **235**, 1387-1390.
7. Dunham, I., *et al.* (1989) *Immunology of HLA, Vol. II*, 73-74.
8. McClelland, M., *et al.* (1987) *Nucl. Acids Res.* **15**, 5985-6005.

Restriction Enzymes and Linkers

Table 14. Enzymes Used to Infrequently Cleave Bacterial Genomes.

Enzyme	Recognition Sequence	Genome G+C% (1)	Average Fragment Size (2) (kb)
***Csp* I**	CGG(A/T)CGG	<40% (*S. aureus*)	400
***Sac* II**	CCGCGG		300
***Sma* I**	CCCGGG		100
***Eco52* I**	CGGCCG		100
***Eco47* III**	AGCGCT		<50
***Not* I**	GCGGCCGC	40-50% (*M. bovis*)	>400
***Sfi* I**	GGCCN$_5$GGCC		>>500
Avr II	CCTAGG		N/D
***Spe* I**	ACTAGT		100
***Bgl* I**	GCCN$_5$GGC		100
***Spo* I**	TCGCGA		100
***Xba* I**	TCTAGA		100
***Nhe* I**	GCTAGC		50
***Ssp* I**	AATATT	>65% (*R. capsulatus*)	>20
***Dra* I**	TTTAAA		N/D

References:

1. McClelland, M., *et al.* (1987) *Nucl. Acids Res.* **15**, 5985-6005.
2. Hung, L. and Bandziulis, R. (1990) unpublished results, Promega Corporation.

Table 15. Linker Sequences.

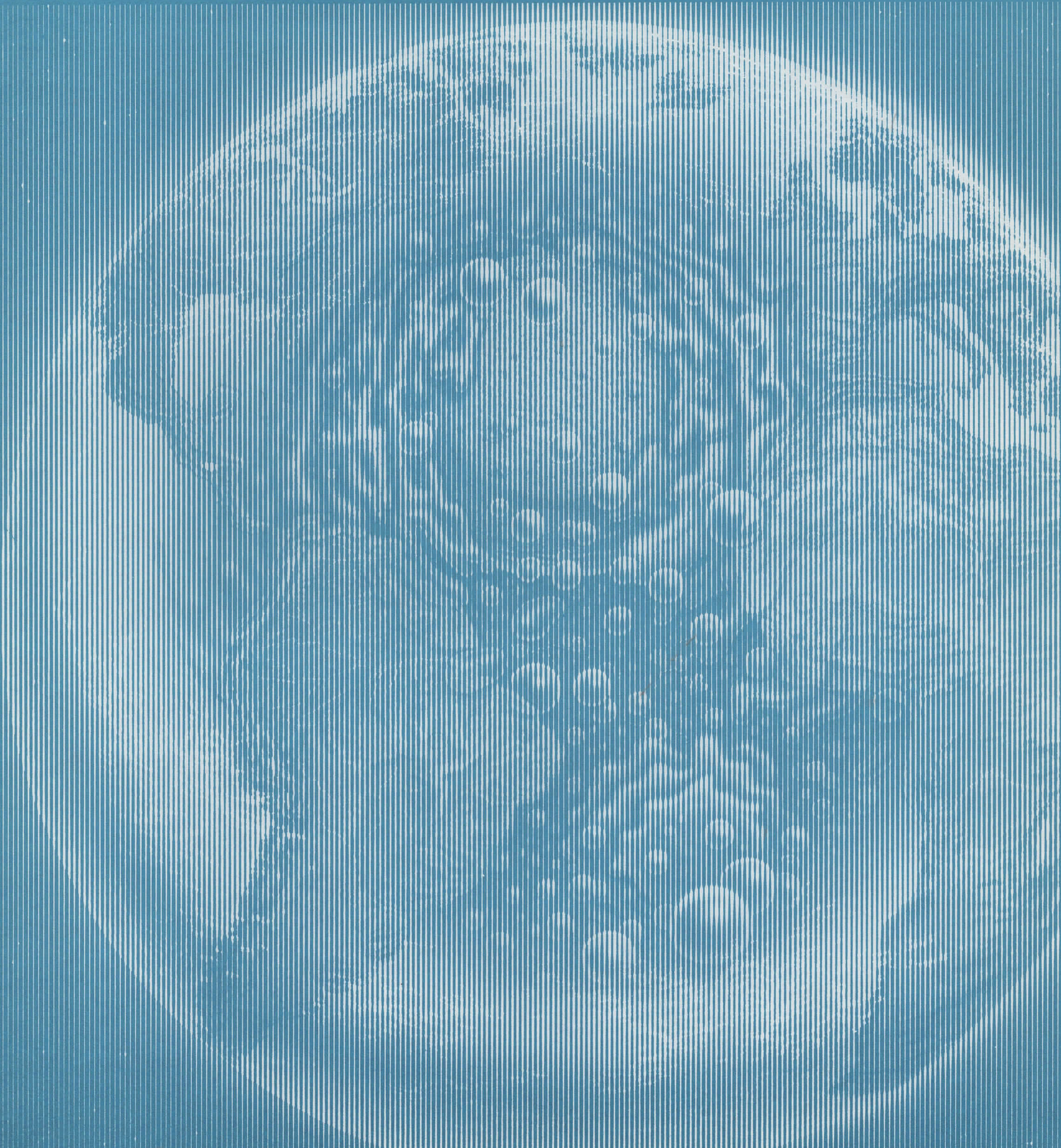
This table lists our selection of linkers synthesized for various cloning strategies. Each linker is available from Promega in both a phosphorylated and non-phosphorylated form and is tested for self-ligation and recutting by the appropriate restriction enzyme.

Product	Sequence	Size
Aat II	5′-d(pGGACGTCC)-3′	8mer
*Bam*H I	5′-d(pCGGATCCG)-3′	8mer
*Bam*H I	5′-d(pCGGGATCCCG)-3′	10mer
*Bam*H I	5′-d(pCGCGGATCCGCG)-3′	12mer
Cla I	5′-d(pCATCGATG)-3′	8mer
Cla I	5′-d(pCCATCGATGG)-3′	10mer
Cla I	5′-d(pCCCATCGATGGG)-3′	12mer
*Eco*R I	5′-d(pGGAATTCC)-3′	8mer
*Eco*R I	5′-d(pCGGAATTCCG)-3′	10mer
*Eco*R I	5′-d(pCCGGAATTCCGG)-3′	12mer
*Hin*d III	5′-d(pCAAGCTTG)-3′	8mer
*Hin*d III	5′-d(pCCAAGCTTGG)-3′	10mer
*Hin*d III	5′-d(pCCCAAGCTTGGG)-3′	12mer
Kpn I	5′-d(pGGGTACCC)-3′	8mer
Mlu I	5′-d(pGACGCGTC)-3′	8mer
Mlu I	5′-d(pCGACGCGTCG)-3′	10mer
Nde I	5′-d(pCCATATGG)-3′	8mer
Not I	5′-d(pGCGGCCGC)-3′	8mer
Not I	5′-d(pAGCGGCCGCT)-3′	10mer
Not I	5′-d(pTTGCGGCCGCAA)-3′	12mer
Pst I	5′-d(pGCTGCAGC)-3′	8mer
Sac I	5′-d(pCGAGCTCG)-3′	8mer
Sal I	5′-d(pGGTCGACC)-3′	8mer
Sma I	5′-d(pCCCCGGGG)-3′	8mer
Sph I	5′-d(pGGCATGCC)-3′	8mer
Xba I	5′-d(pCTCTAGAG)-3′	8mer
Xba I	5′-d(pGCTCTAGAGC)-3′	10mer
Xba I	5′-d(pTGCTCTAGAGCA)-3′	12mer
Xho I	5′-d(pCCTCGAGG)-3′	8mer
Xho I	5′-d(pCCCTCGAGGG)-3′	10mer
Xho I	5′-d(pCCGCTCGAGCGG)-3′	12mer

Restriction Enzymes and Linkers

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Plasmid Cloning and Transcription *in vitro*

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Plasmid Cloning and Transcription *in vitro*

Contents
(continued)

I. General Considerations

The Riboprobe® and Riboprobe® Gemini systems are convenient vector/enzyme systems for the preparation *in vitro* of high specific activity single-stranded RNA probes or microgram quantities of defined RNA transcripts from cloned DNA inserts. The complete Riboprobe systems include the specified vectors, RNA polymerases, and all of the required reagents (excluding radioisotope) for performing transcription reactions *in vitro*. In addition to the vectors supplied with the complete Riboprobe systems, all other vectors in the Riboprobe series may be used for transcription of RNA.

A. Properties of Individual Riboprobe® Vectors

The features and applications of Promega's Riboprobe vectors are summarized in Tables 1 and 2 (pgs. 49-50). Plasmid maps and multiple cloning site sequences are provided in the Vector Maps chapter, pg. 311. The pSP64-pSP73 series of vectors are general-purpose cloning vectors which allow the synthesis *in vitro* of RNA transcripts. The pSP64, pSP65, and pSP64(polyA) vectors allow RNA to be transcribed from one strand of the cloned DNA using the SP6 promoter. The pSP70-pSP73 vectors and all pGEM®* vectors contain dual opposed SP6 and T7 promoters flanking the multiple cloning site, allowing RNA to be transcribed from either strand of the insert. The pSP64(polyA) vector contains a stretch of dA:dT residues at one end of the multiple cloning site, allowing the synthesis *in vitro* of RNA containing a synthetic 30 base poly(A) tail.

The pGEM-3Z and pGEM-4Z vectors were the first Riboprobe vectors to incorporate blue/white screening for recombinants. These are general-purpose cloning vectors, containing versatile multiple cloning regions and allowing synthesis of RNA transcripts from either strand using the SP6 and T7 RNA polymerase promoters. These vectors are differentiated by the orientation of their multiple cloning region. The subsequently developed pGEM-3Zf(+/–) vectors include the origin of replication of the filamentous bacteriophage f1 to allow production of single-stranded DNA. The orientation of the f1 origin (either + or –) determines which of the plasmid DNA strands will be secreted as single-stranded DNA.

Other pGEM-Zf vectors include the aforementioned features plus unique multiple cloning regions designed for specific applications. The pGEM-5Zf(+/–) and pGEM-7Zf(+/–) plasmids are distinct in that their multiple cloning sites contain a central cluster of restriction sites which generate 5′ overhangs flanked by sites which generate 3′ overhangs. This configuration is designed for use with the Erase-a-Base® system, in which a nested set of unidirectional deletions is generated for sequence analysis of large DNA fragments.

The pGEM-9Zf(–) and pGEM-11Zf(+/–) plasmids are distinguished by their multiple cloning sites, which include restriction sites (*Sfi* I, *Eco*R I, and *Not* I) that can be used in direct cDNA cloning strategies or for convenient subcloning of cDNAs cloned into the λgt11 *Sfi-Not* vector. The pGEM-13Zf(+) plasmid can be used for subcloning and in-frame expression of fusion proteins initially cloned into the λgt11 *Sfi-Not* vector.

The pGEMEX™ vectors are optimized for expression of large quantities of T7 gene 10 fusion proteins from the T7 promoter. RNA transcripts can also be synthesized from either strand of the insert DNA using the SP6 and T3 promoters flanking the multiple cloning site.

All pGEM-Z vectors are available separately or as components of specific systems.

* Patent No. 4,766,072

I. General Considerations

(continued)

B. Applications of the Riboprobe Series of Vectors RNA Transcripts

Transcription of RNA from the Riboprobe plasmids is performed with SP6 and T7 RNA polymerase and, for the pGEMEX plasmids, T3 RNA polymerase. Because these polymerases are extremely promoter-specific (i.e., there is no transcription cross-talk), virtually homogeneous RNA can be obtained using plasmid DNA as the template in a transcription reaction (1). When it is desirable to copy only insert DNA sequences, the plasmid is linearized at an appropriate restriction site prior to the transcription reaction and only discrete "runoff" transcripts are obtained, virtually free of vector sequences (Figure 1, pg. 48). RNA transcripts may be used to generate probes for hybridization to Northern and Southern blots, plaque and colony lifts, tissue sections, and chromosome spreads. RNA transcripts are also useful for S1 nuclease mapping, mRNA synthesis for translation *in vitro*, and generation of antisense RNAs to block translation. These applications are discussed in more detail on pg. 65 and in the references cited on pgs. 73-74.

Blue/White Color Screening

The pGEM-Z and pGEM-Zf series of vectors were the first SP6/T7 vectors to incorporate blue/white color selection for insertion. These vectors contain a sequence coding for the *lac* α-peptide, interrupted by a multiple cloning site. Nonrecombinant plasmids produce a functional α-peptide which, by complementing the product of the host cell *lac*ZΔM15 gene, leads to production of functional β-galactosidase. Bacterial colonies harboring the *lac*ZΔM15 gene on an F′ and also containing a pGEM-Z vector are blue in color when plated on indicator media containing IPTG and X-Gal. However, when the *lac* α-peptide is disrupted by cloning into the pGEM-Z multiple cloning region, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM-Z vector constructs are white.

Sequencing

The SP6, T7, and T3 RNA polymerase promoters flanking the Riboprobe plasmid multiple cloning sites also serve as convenient and specific priming sites for sequencing reactions. By selecting a given sequencing primer, either strand of a DNA insert can be quickly sequenced from a single double-stranded plasmid template. If necessary, single-stranded DNA may be generated for sequence analysis using the pGEM-Zf plasmids. When sequence must be obtained more than 500 bases from the end of the insert, the Erase-a-Base system may be used to generate a nested set of exonuclease III deletions in the insert DNA, effectively moving the priming sites closer to the sequence of interest (2). The pGEM-5Zf(+/–) and pGEM-7Zf(+/–) vectors were constructed for use with the Erase-a-Base system. The multiple cloning regions in these vectors contain sites for restriction enzymes that produce 5′ overhangs or blunt ends (sensitive to exonuclease III). These enzymes sites are flanked on both sides by blocks of restriction sites that generate 3′ overhangs (resistant to exonuclease III). This arrangement allows exonuclease III deletions to proceed into the insert DNA while preserving the desired sequencing priming site. Further information on sequencing systems and the Erase-a-Base system is provided on pgs. 75-121.

Single-Stranded DNA Production

Vectors in the pGEM®-Zf series contain the origin of replication for the filamentous phage f1, and thus can be used to generate single-stranded DNA (ssDNA) for sequencing or mutagenesis *in vitro*. For induction of ssDNA, bacterial cells containing pGEM-Zf recombinants are infected with an appropriate helper phage. The plasmid then enters the f1 replication mode and the resulting ssDNA is exported from the cell as an encapsidated virus-like particle. The single-stranded plasmid DNA is purified from the supernatant by simple precipitation and extraction procedures. The orientation of the f1 origin (either + or –) determines which of the strands of the plasmid will be secreted. Promega offers four systems for ssDNA production, based on the pGEM-3Zf(+/–), pGEM-5Zf(+/–), pGEM-7Zf(+/–), and pGEM-11Zf(+/–) vectors. Each single strand system contains the specified pair of pGEM-Zf plasmids containing the f1 origin in opposite orientations plus two host strains and two helper phage stocks, allowing optimization of ssDNA production for individual recombinants. Refer to pg. 109 for details of ssDNA production.

Plasmid Cloning and Transcription *in vitro*

I. General Considerations

(continued)

High-Level Expression

The pGEM Express systems, based on the T7 expression system developed by Studier (3), employ a convenient vector/host combination for high level expression of cloned genes *in vivo*. Sequences cloned into the pGEMEX vectors (see pgs.362-364 for maps) are specifically expressed as T7 gene 10 fusion proteins in JM109(DE3), a specially constructed host strain which contains an IPTG-inducible gene for T7 RNA polymerase. The bacteriophage T7 gene 10 leader peptide (260 amino acids) is very efficiently expressed in this host.

The pGEMEX expression vectors are unique in that they contain three RNA polymerase promoters. In each, the T7 promoter and the gene 10 ribosome binding site are positioned upstream from the gene 10 leader fragment. In addition, dual opposed SP6 and T3 promoters flank the multiple cloning region, allowing the production *in vitro* of high specific activity single-stranded RNA probes and substrate amounts of pure RNA from either strand of the cloned insert. A T7 transcription terminator is incorporated downstream from the multiple cloning region.

In-frame subcloning from lambda expression vectors into the pGEMEX plasmid vectors is easily performed. The reading frame of the pGEMEX-1 vector at the *Eco*R I cloning site is identical to that of λgt11, and the reading frame of the pGEMEX-2 vector at the *Sfi* I cloning site is identical to that of the λgt11 *Sfi-Not* directional cloning vector. Since the cloning sites in the two pGEMEX vectors are in different reading frames, these vectors may be used to clone DNA restriction fragments in two of the three possible reading frames

Site-Directed *in vitro* Mutagenesis

The Altered Sites™ *in vitro* mutagenesis system consists of a unique mutagenesis vector and a simple, straightforward procedure for selection of oligonucleotide-directed mutants. The system is based on the use of a second mutagenic oligonucleotide to confer antibiotic resistance to the mutant DNA strand. The system employs a phagemid vector, the pSELECT™-1 plasmid, which contains two genes for antibiotic resistance. The gene for tetracycline resistance is always functional. The gene for ampicillin resistance has been inactivated. An oligonucleotide is provided which restores ampicillin resistance to the mutant strand

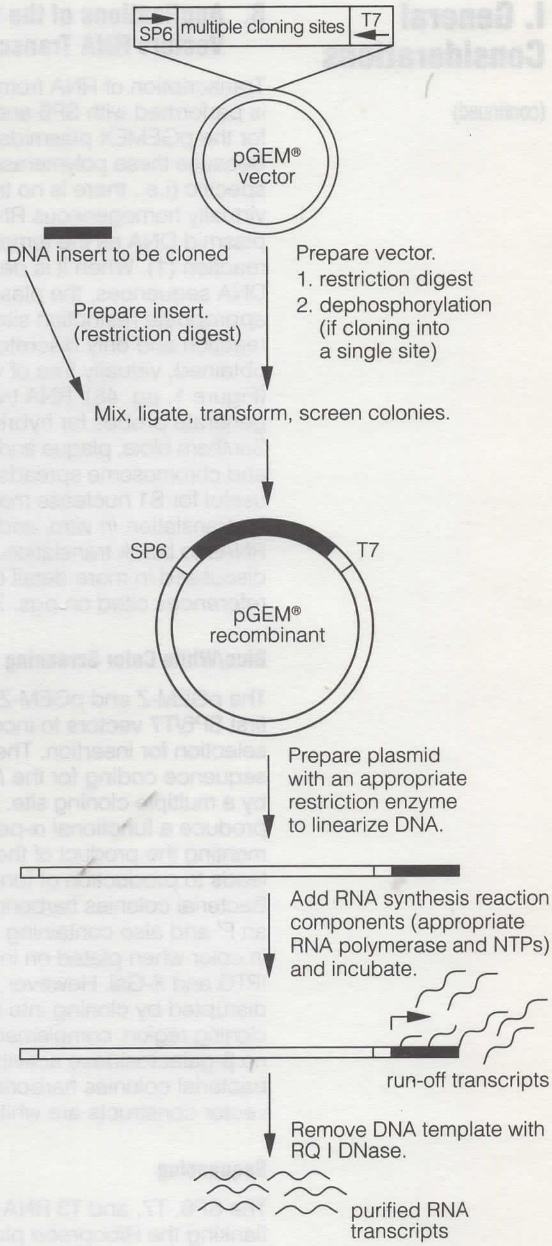

Figure 1. Schematic diagram of the Riboprobe Gemini system.

Promega

I. General Considerations

(continued)

during the mutagenesis reaction. This oligonucleotide is annealed to the single-stranded DNA template at the same time as the mutagenic oligonucleotide and subsequent synthesis and ligation of the mutant strand links the two. The DNA is transformed into a repair minus strain of *E. coli* (BMH 71-18 mut S) and the cells are grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109 or a similar host ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants.

The pSELECT-1 vector contains a large multiple cloning site flanked by the SP6 and T7 promoters. These sites may be used as convenient priming sites for sequencing and for generation of high specific activity RNA probes from either strand of the insert DNA. Further information on this system is provided on pg. 98.

Table 1. Features and Applications of Riboprobe, Riboprobe Gemini, pGEMEX, and pSELECT Vectors.

	pSP64, pSP65 & pSP64(polyA)	pSP70 & pSP71	pSP72 & pSP73	pGEM-1 & pGEM-2	pGEM-3 & pGEM-4	pGEM-3Z & pGEM-4Z	pGEM-3Zf(+) & pGEM-3Zf(–)	pGEM-5Zf(+) & pGEM-5Zf(–)	pGEM-7Zf(+) & pGEM-7Zf(–)	pGEM-9Zf(–)	pGEM-11Zf(+) & pGEM-11Zf(–)	pGEM-13Zf(+)	pGEMEX-1 & pGEMEX-2	pSELECT-1
Size (bp)	2,999 3,005 3,033	2,417 2,419	2,642 2,464	2,865 2,869	2,867 2,871	2,743 2,746	3,199 3,199	3,003	3,000	2,925	3,223	3,181	3,995 3,997	5,680
Ampicillin selection	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Promoters	S	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T3,T7	S,T7
Blue/white screening for recombinants	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	N	Y
Single-stranded DNA production	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
Direct dsDNA sequencing possible	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sequencing primers applicable	S	S,T7	S,T7	S,T7	S,T7	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 T3	S,T7 M
Classification	R	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG

Key: R - Riboprobe
RG - Riboprobe Gemini
S - SP6
M - M13

Note: "pGEM" is a registered trademark of Promega Corporation and should be referred to as pGEM® vector.

Plasmid Cloning and Transcription *in vitro*

I. General Considerations

(continued)

Table 2. Summary of Restriction Sites in Multiple Cloning Regions of Riboprobe, Riboprobe Gemini, pGEMEX, and pSELECT Vectors.

	pSP64, pSP65 & pSP64(polyA)	pSP70 & pSP71	pSP72 & pSP73	pGEM-1 & pGEM-2	pGEM-3 & pGEM-4	pGEM-3Z & pGEM-4Z	pGEM-3Zf(+) & pGEM-3Zf(−)	pGEM-5Zf(+) & pGEM-5Zf(−)	pGEM-7Zf(+) & pGEM-7Zf(−)	pGEM-9Zf(−)	pGEM-11Zf(+) & pGEM-11Zf(−)	pGEM-13Zf(+)	pGEMEX-1 & pGEMEX-2	pSELECT-1	
Aat II								●	●						*Aat* II
Acc I	●		●	●	●	●	●				●		●		*Acc* I
Ava I	●			●	●	●	●								*Ava* I
Apa I								●	●		●		●		*Apa* I
*Bam*H I	●		●	●	●	●	●		●		●		●	●	*Bam*H I
Bgl II		●	●												*Bgl* II
BstX I								●	●						*BstX* I
Cla I		●	●						●						*Cla* I
Csp45 I									●						*Csp45* I
Eag I								●			●		●		*Eag* I
*Eco*R I	●	●	●	●	●	●	●		●	●	●		●	●	*Eco*R I
*Eco*R V		●	●					●							*Eco*R V
Hind II	●			●	●	●	●				●		●		*Hind* II
Hind III	●	●	●	●	●	●	●		●	●	●	●	●	●	*Hind* III
Kpn I			●		●	●	●		●					●	*Kpn* I
Nco I								●							*Nco* I
Nde I								●							*Nde* I
Not I								●		●	●	●	●		*Not* I
Nsi I								●	●	●	●		●		*Nsi* I
Pst I	●		●	●	●	●	●	●						●	*Pst* I
Pvu II		●	●												*Pvu* II
Sac I	●		●	●	●	●	●	●	●	●	●		●	●	*Sac* I
Sac II								●							*Sac* II
Sal I	●		●	●	●	●	●	●		●	●		●	●	*Sal* I
Sfi I										●	●	●	●		*Sfi* I
Sph I (*Bbu* I)			●		●	●	●	●	●		●		●	●	*Sph* I (*Bbu* I)
Sma I	●		●	●	●	●	●		●					●	*Sma* I
Spe I								●		●					*Spe* I
*Tth*111 I										●					*Tth*111 I
Xba I	●		●	●	●	●	●		●	●	●		●	●	*Xba* I
Xma I									●						*Xma* I
Xho I		●	●						●		●		●		*Xho* I

Note: "pGEM" is a registered trademark of Promega Corporation and should be referred to as pGEM® vector.

II. Cloning of DNA Inserts

A. Preparation of Vector and Insert DNA for Cloning

Both fragment and vector DNA should be digested with appropriate restriction enzymes to generate compatible ends for cloning. If a single restriction enzyme is used to prepare the vector, the DNA can be treated with calf intestinal alkaline phosphatase (CIAP) to remove 5′ phosphate groups and thus prevent recircularization of the vector during ligation.

A sufficient amount of DNA should be prepared to allow for control reactions for the digestion, ligation, and transformation steps.

To ensure that the correct restriction fragment is cloned, the insert DNA can be purified by electrophoresis on an acrylamide or low melting point agarose gel and then eluted from the gel. Alternatively, unfractionated restriction fragments can be cloned into the target plasmid and the recombinant of the desired size can then be identified by gel electrophoresis. Useful references for the purification of DNA fragments are listed on pg. 74.

Reagents to be Supplied by the User

- 1% agarose minigel
- 0.5M EDTA
- TE-saturated phenol/chloroform (pg. 57)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)

Protocol

1. Prepare the insert and vector DNAs by restriction endonuclease digestion. The following 50µl reaction is provided as an example.

   a. Add the following components:

plasmid or insert DNA	5µg
appropriate restriction enzyme 10X buffer	5µl
1mg/ml acetylated BSA (optional)	5µl
appropriate restriction enzyme	5-25u
deionized H_2O	to final volume 50µl

   b. Incubate at the appropriate temperature for 1-3 hours.

   c. Check that the reaction has gone to completion by electrophoresis of a sample (0.3µg) on a 1% agarose minigel.

2. If the vector has been digested with a single restriction endonuclease, the DNA should be treated with calf intestinal alkaline phosphatase (CIAP) as follows:

   a. Add the following components directly to the digested DNA:

CIAP 10X buffer (pg. 57)	10µl
CIAP	0.01u/pmol ends
deionized H_2O	to final volume 100µl

   b. Incubate for 60 minutes at 37°C.

   c. To stop the reaction, add 2µl of 0.5M EDTA.

   Note: CIAP can interfere with the efficiencies of ligation and transformation reactions and should therefore be removed from the reaction mixture prior to ligation of the phosphatase-treated vector to the insert DNA (see Steps 3-6, below).

3. Add 1 volume of TE-saturated phenol/chloroform (pg. 57), vortex for 1 minute, and centrifuge at 12,000 x g for 2 minutes.

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 3. Repeat this step.

5. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate. Add 2 volumes of ethanol and leave at –70°C for 30 minutes. Collect the DNA pellet by centrifugation at 12,000 x g for 5 minutes.

6. Carefully pour off the supernatant, wash the pellet with 1ml of 70% ethanol, dry briefly in a vacuum desiccator. Resuspend the pellet in 15-20µl of nuclease-free H_2O. Determine the approximate DNA concentration by absorption spectroscopy or by agarose gel electrophoresis and comparison to known amounts of DNA standards.

Plasmid Cloning and Transcription *in vitro*

II. Cloning of DNA Inserts

(continued)

B. Ligation of Vector and Insert DNA

To obtain the optimal ratio of vector to insert DNA, a 1:1, 1:3 and a 3:1 molar ratio of vector:insert should be tried when cloning a fragment into one of the Riboprobe® system plasmid vectors. Table 3 illustrates the conversion of molar ratios to mass ratios for a 3.0kb plasmid and a 0.5kb DNA fragment which is to be inserted.

Protocol

1. Set up a ligation reaction as described below. This example assumes a 1:1 vector:insert ratio.

vector DNA	100ng
insert DNA	17ng
T4 DNA ligase (Weiss units)	1u
ligase 10X buffer (pg. 57)	1μl
deionized H_2O	to final volume 10μl

Ligation reactions should be performed according to the following guidelines for incubation temperature and time:

4°C,	overnight
or 15°C,	4-6 hours
or 25°C,	1 hour

2. Following the ligation reaction, transform the plasmid DNA into competent cells of an appropriate host strain (see Sections C and D, below).

Note: To monitor the efficiency of the ligation and transformation steps, host cells should also be transformed with uncut, nonrecombinant vector DNA as well as nonrecombinant vector DNA which has been cut, phosphatase-treated, and ligated.

Table 3. Calculation of Vector and Insert DNA Ratios. In this example, the mass ratio of the vector to the insert is 3.0:0.5 or 6:1.

	Vector	Insert
1:1 molar ratio	1.0μg	0.167μg
1:3 molar ratio	1.0μg	0.501μg
3:1 molar ratio	1.0μg	0.056μg

C. Transformation Protocols

Many different transformation protocols have been successfully applied to these vectors (4,5,6). The host strain used should be compatible with the method to be used to select transformants. Refer to Sections D.1 and D.2 for the conditions to be used for blue/white color screening and standard ampicillin selection. Strains to be used for color selection (such as JM109) should always be grown on minimal plates (M-9) supplemented with thiamine-HCl prior to preparation of competent cells and transformation. This selects for the presence of the F′ which is necessary for color selection. Competent cells of a variety of bacterial strains are available from Promega. For those preferring to prepare their own, a method is provided below for the large scale preparation of competent cells suitable for long-term storage at –80°C.

Reagents to be Supplied by the User

- M-9 plates + thiamine-HCl (pg. 57)
- LB medium (pg. 57)
- trituration buffer (pg. 57)
- 80% glycerol

1. Preparation of Competent Cells

a. Streak the desired bacterial strain on selective plates overnight. If blue/white color selection is to be used, streak JM109 or another appropriate host strain on M-9 plates containing thiamine-HCl (pg. 57).

b. Inoculate 25ml of LB medium (pg. 57) with a single colony. Incubate at 37°C overnight with vigorous shaking.

c. Using a 2.8 liter flask, inoculate 500ml of LB medium with 5ml of cells from the overnight culture.

d. Shake the culture at 150-200rpm at 37°C until the A_{600} reaches 0.45-0.55.

e. Chill the cells in ice water for 2 hours and collect by centrifugation at 2,500 x g for 15-20 minutes at 4°C.

f. Resuspend the cells in 10-20ml of ice-cold trituration buffer (pg. 57) and then dilute to 500ml with the same solution.

II. Cloning of DNA Inserts

(continued)

g. Incubate the cells on ice for 45 minutes.

h. Centrifuge the cells at 1,800 x g for 10 minutes and gently resuspend in 50ml of ice-cold trituration buffer.

i. Pool the cells and add 80% glycerol dropwise with gentle swirling to a final concentration of 15% (v/v). Aliquot the cells in 0.2-1.0ml quantities, freeze on dry ice, and store at –80°C.

2. Transformation of Competent Cells

a. Thaw on ice a 200µl aliquot of JM109 or other competent cells (available from Promega).

b. Add 3µl of DMSO to the competent cells, mix briefly, and add 10-20ng of plasmid DNA. **Note:** DMSO should be frozen in aliquots and not reused once thawed.

c. Incubate the cells on ice for 30 minutes.

d. **Optional:** For some strains, a heat shock at 42°C for 1-2 minutes after the incubation on ice has been reported to increase transformation efficiency. Following a heat shock, cool the tube on ice for 1 minute.

e. Add 2ml of LB medium and shake gently at 37°C for 1 hour to allow the cells to recover.

f. The cells can be plated directly or concentrated before plating. To concentrate the cells, centrifuge the culture for 1 minute in a microcentrifuge.

g. Pour off the supernatant and resuspend the cells in each tube in 100-200µl of LB medium.

h. Plate the cells on an LB plate containing 50µg/ml ampicillin and incubate at 37°C for 12-14 hours.

D. Selection for Transformants

The JM109 and several other bacterial host strains are suitable for transformation and ampicillin selection with all of Promega's plasmids. Since pGEM® plasmids are supplied as modified DNA, hosts may be either restriction$^+$ or restriction$^-$. The use of a *rec*A$^-$ host such as JM109 is preferred, however, since this prevents undesirable recombination between the insert and the host chromosomal DNA.

Reagents to be Supplied by the User

Instructions for preparing these reagents are provided on pg. 57.

- M-9 plates + thiamine-HCl
- LB plates + ampicillin
- IPTG stock solution
- X-Gal stock solution
- cracking 2X buffer
- 1% agarose gel + 0.5µg/ml ethidium bromide

1. Blue/White Color Screening

For blue/white color screening of the pGEM-Z and pGEM-Zf plasmids, host strains such as JM109 or NM522 must be used. These carry *lac*ZΔM15 and lacI^Q on an F′ episome. Strains containing an F′ episome should always be maintained on minimal plates (M-9) supplemented with thiamine-HCl. This selects for the presence of the F′ which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positives. Genotype information and more complete descriptions of these strains are provided on pgs. 54 and 410.

To use the blue/white color screening for recombinants, plate transformed JM109 or NM522 cells on LB plates (pg. 57) containing 50µg/ml ampicillin, 0.5mM IPTG, and 40µg/ml X-Gal (pg. 57). Incubate the plates overnight at 37°C. Recombinant colonies will be white.

(continued on next page)

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(continued)

An alternative to preparing plates containing IPTG and X-Gal is to spread plates with 20µl of 50mg/ml X-Gal and 100µl of 100mM IPTG (pg. 57) and allow these components to absorb for 30 minutes at 37°C prior to plating cells.

Note: In general, colonies containing β-galactosidase activity grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies are pinpoint in size, while the white colonies are approximately a millimeter in size.

2. Ampicillin Selection

Blue/white screening is not available with the pGEMEX™ vectors and with Promega's older vectors, including the pSP64/65, pSP70/71, pSP72/73, and pGEM®-1, -2, -3 and -4 vectors. To select for transformants using these plasmids, plate transformed cells on media containing 50µg/ml ampicillin and incubate overnight at 37°C.

To screen ampicillin-selected colonies for recombinants, it is necessary either to prepare plasmid DNA for restriction analysis (see Section E, below) or to rapidly estimate the size of plasmid DNA without endonuclease digestion (14) using the cracking procedure described below.

Cracking Procedure

a. Using sterile toothpicks or pipet tips, pick individual colonies from plates and smear each near the bottom of a microcentrifuge tube containing 50µl of 10mM EDTA, pH 8.0.

b. Add 50µl of freshly made cracking 2X buffer (pg. 57). Resuspend the cells by vortexing.

c. Incubate the sample at 70°C. Allow to cool to room temperature.

d. Add 1.5µl of 4M KCl and 0.5µl of 0.4% bromophenol blue and vortex.

e. Place on ice for 5 minutes.

f. Microcentrifuge for 3 minutes at 4°C.

g. Run 25-50µl of the supernatant on a 0.7% agarose gel.

h. After the dye has migrated 3/4 the length of the gel, stain the gel by soaking it for 30-40 minutes in a solution of ethidium bromide (0.5µl/ml in water). Using appropriate supercoiled DNA markers, the plasmid sites can be estimated.

Table 4. Common Bacterial Strains Used with Promega's pGEM® Plasmids.

The following is a partial list of common bacterial strains used in conjunction with Promega's pGEM® plasmids. An **a** indicates that these strains can be utilized with the pGEM®-Z series of vectors on media containing IPTG and X-Gal to distinguish recombinants. A **b** indicates that these strains can be utilized with the pGEM®-Zf series of vectors to produce single-stranded plasmid DNA using the appropriate filamentous helper phage. Competent cells available from Promega are listed in bolded type.

Strain	Genotype
C600 (8)	F⁻, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44, λ⁻
DH5α (8)[a]	F⁻, φ80d*lac*ZΔM15, *end*A1, *rec*A1, *hsd*R17 (r_K^-,m_K^+), *sup*E44, *thi*-1, *gyr*A96, *rel*A1, Δ(*lac*ZYA-*arg*F), U169, λ⁻
HB101 (7)	F⁻, *thi*-1, *hsd*S20 (r_B^-,m_B^-), *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (str^r), *xyl*-5, *mtl*-1, *sup*E44, λ⁻
JM101 (9)[a,b]	*sup*E, *thi*, Δ(*lac*-*pro*AB), [F′, *tra*D36, *pro*AB⁺, *lac*I^{q}ZΔM15], λ⁻
JM109 (10)[a,b]	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17 (r_K^-,m_K^+), *sup*E44, Δ(*lac*-*pro*AB), *rel*A1, [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15
MB408 (11)	F⁻, *rec*F, *rec*B21, *rec*C22, *sbc*B15, *hfl*A, *hfl*B, *hsd*R, (tet^r)
NM522 (13)[a,b]	*sup*E, *thi*, Δ(*lac*-*pro*AB), Δ*hsd*5 (r⁻, m⁻), [F′, *pro*AB, *lac*I^{q}ZΔM15]
TB1 (12)[a]	F⁻, *ara*Δ(*lac*-*pro*AB), *rps*L, *hsd*R17 (r_K^-,m_K^+), φ80d*lac*ZΔM15

II. Cloning of DNA Inserts

(continued)

E. Isolation of Plasmid DNA

This plasmid mini-prep protocol allows for the rapid isolation of small amounts of plasmid DNA without the need for column purification or banding in CsCl gradients (15). The purified DNA is suitable for use as template DNA for *in vitro* transcription reactions. The procedure takes advantage of the rapid alkaline denaturation of plasmid and chromosomal DNA and the selective renaturation of plasmid DNA following neutralization of the solution. The pGEM plasmids are high copy number plasmids, with approximately 300-400 copies per cell. Using this protocol, 1-4µg of DNA can be obtained from 1.5ml of culture. The procedure may be scaled up when larger amounts of DNA are needed.

Reagents to be Supplied by the User

Instructions for preparing these reagents are provided on pg. 57.

- LB medium + 50µg/ml ampicillin
- miniprep lysis buffer
- 0.2N NaOH, 1% SDS (prepare fresh)
- potassium acetate solution, pH 4.8
- DNase-free RNase A (see pg. 57 for preparation)
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)

Optional:
- 4M NaCl
- 13% polyethylene glycol (MW 6,000-8,000) in H_2O

Protocol

1. Inoculate 5ml of LB medium (pg. 57) containing 50µg/ml ampicillin with a single bacterial colony. Incubate at 37°C overnight with vigorous shaking.
2. Place 1.5ml of the overnight culture into a micro-centrifuge tube and centrifuge at 12,000 x g for 1 minute. The remainder of the overnight culture can be stored at 4°C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
4. Resuspend the pellet by vortexing in 100µl of ice-cold miniprep lysis buffer (pg. 57).
5. Incubate for 5 minutes at room temperature.
6. Add 200µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS. Mix by inversion. **DO NOT VORTEX**. Incubate for 5 minutes on ice.
7. Add 150µl of ice-cold potassium acetate solution, pH 4.8 (pg. 57). Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.
8. Centrifuge at 12,000 x g for 5 minutes.
9. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
10. Add RNase A (see pg. 57 for preparation) to a final concentration of 20µg/ml.
11. Incubate at 37°C for 20 minutes.
12. Add one volume of TE-saturated phenol/chloroform (pg. 57). Vortex for 1 minute and centrifuge at 12,000 x g for 2 minutes.
13. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in step 12.
14. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of ethanol. Mix and allow the sample to precipitate 5 minutes on dry ice.
15. Centrifuge at 12,000 x g for 5 minutes, remove the supernatant, and wash the pellet with 1ml of prechilled 70% ethanol. Dry the pellet under vacuum.
16. Dissolve the dried pellet in 10-20µl of sterile deionized water.

Plasmid Cloning and Transcription *in vitro*

II. Cloning of DNA Inserts

(continued)

F. Troubleshooting

Symptoms	Possible Causes	Comments
Few or no transformed colonies.	Inactive competent cells.	Competent cells may exhibit lower transformation efficiencies 5-6 weeks after preparation. To verify that bacteria are competent, perform a test transformation using a known amount of a standard supercoiled plasmid DNA.
	Unsuccessful ligation.	Analyze samples of: A) linearized vector DNA and B) ligated vector and DNA fragment on a 0.8% agarose gel. If ligation was successful, the banding pattern of the ligation products should be different than that of the unligated sample.
	Excess ligation products added to competent cells.	The volume of the ligation products added should not exceed 5% of the volume of the competent cells.
No ligation.	Inactive T4 DNA ligase.	Verify that the T4 DNA ligase is active by attempting to ligate a linearized plasmid DNA.
	Inactive T4 DNA ligase buffer.	T4 DNA ligase 10X buffer should be stored in aliquots at –20°C and used only once after thawing. Ligase buffer stored at 4°C or subjected to multiple freeze-thaw cycles tends to be inactive for ligation reactions due to degradation of ATP.
	Digested vector termini are not compatible with the fragment.	Under certain circumstances (e.g., close proximity of restriction sites in the multiple cloning region) complete digestion of the vector DNA with two different restriction enzymes is difficult to attain. To determine if double digestion of the target vector is possible, perform a digestion/labeling procedure as described in chapter one of reference 5.
High background of nonrecombinants.	Unsuccessful dephosphorylation of vector DNA.	Attempt to religate the linearized dephosphorylated vector DNA. Dephosphorylated vector DNA should be religated with very low efficiency. Alternatively, verify the activity of the phosphatase by performing a test dephosphorylation of a ^{32}P-5′-end labeled DNA molecule. Precipitate with 10% trichloroacetic acid and 1% Na pyrophosphate to separate the test DNA from released ^{32}P (16).
	Medium lacks the correct antibiotic.	Perform a mock transformation with no DNA added. If colonies grow, the medium should be discarded.
	Ratio of linearized vector to insert DNA is too high.	Reduce the amount of linearized vector DNA in the reaction. When the ratio of linearized vector to insert is too high, religation of the vector is favored.

II. Cloning of DNA Inserts

(continued)

Composition of Solutions

CIAP 10X buffer:

500mM	Tris-HCl, pH 9.0
10mM	$MgCl_2$
1mM	$ZnCl_2$
10mM	spermidine

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Ligase 10X buffer:

300mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

Note: We recommend that ligase 10X buffer be stored in aliquots at -20°C to avoid multiple freeze-thaw cycles. Degradation of ATP in the ligation buffer is the most common reason for failure of simple ligations.

M-9 plates (per liter):

6.0g	Na_2HPO_4
3.0g	KH_2PO_4
0.5g	NaCl
1.0g	NH_4Cl
15g	agarose

Add H_2O to approximately 1 liter. Autoclave. Cool to 50°C. Add:

2.0ml	1M $MgSO_4$
0.1ml	1M $CaCl_2$
10.0ml	20% glucose
1.0ml	1M thiamine-HCl

Potassium acetate solution, pH 4.8:

Prepare 60ml of 5M potassium acetate. Add 11.5ml of glacial acetic acid and 28.5 ml of H_2O. This solution is 3M with respect to potassium and 5M with respect to acetate. Store at 4°C.

DNase-free RNase A:

To make DNase-free RNase A, prepare a 10mg/ml solution of RNase A in 10mM Tris-HCl, pH 7.5, 15mM NaCl. Heat at 100°C for 15 minutes and cool slowly to room temperature.

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust pH to 7.5 with NaOH and autoclave. Where indicated, add ampicillin to 50µg/ml after autoclaved solution has cooled to 50°C.

LB plates with ampicillin (per liter):

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.5 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding ampicillin (50µg/ml final conc.). Pour 30-35ml of medium into 100mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

Trituration buffer:

100mM	$CaCl_2$
70mM	$MgCl_2$
40mM	sodium acetate, pH 5.5

This solution should be prepared fresh and filter-sterilized for each use.

IPTG stock solution (0.1M):

1.2g	IPTG

Add H_2O to 50ml final volume. Filter-sterilize and store at 4°C.

X-Gal stock solution:

X-Gal is available from Promega as a 50mg/ml stock in N,N′ dimethylformamide.

Cracking 2X buffer (per 50ml):

2ml	5M NaOH
2.5ml	10% SDS
10g	sucrose

Add deionized H_2O to 50ml final volume.

Miniprep lysis buffer*:

25mM	Tris-HCl, pH 8.0
10mM	EDTA
50mM	glucose

*Buffer tablets available from Promega.

Plasmid Cloning and Transcription *in vitro*

III. RNA Transcription *in vitro*

A. DNA Template Preparation for Transcription *in vitro*

1. Template Linearization

When the presence of vector sequences on the probe will not interfere with subsequent applications, transcripts can be synthesized using an intact plasmid as the template. Alternatively, the plasmid can be linearized to produce "run-off" transcripts derived from the insert sequence only.

To prepare a plasmid for the production of "run-off" transcripts, linearize the vector with a suitable restriction endonuclease. After the restriction digestion, extract the linearized plasmid with phenol/chloroform before using the DNA for transcription reactions *in vitro*.

It is important that the restriction digestion be performed to completion. A small amount of undigested plasmid DNA can give rise to very long transcripts which may incorporate a substantial fraction of the radiolabeled rNTP.

Extraneous transcripts have been reported to appear (17) in addition to the expected transcript when templates contain 3′ protruding ends (overhangs). The extraneous transcripts can contain sequences complementary to the expected transcript as well as sequences corresponding to vector DNA. Therefore, we recommend that plasmids not be linearized with any of the enzymes listed in Table 5.

Table 5. Restriction Enzymes that Generate 3′ Protruding Ends.

Aat II	*Apa* I	*Ban* II
Bgl I	*Bsp* 1286 I	*Bst*X I
Cfo I	*Hae* II	*Hgi*A I
Hha I	*Kpn* I	*Pst* I
Pvu I	*Sac* I	*Sac* II
Sfi I	*Sph* I	

2. Conversion of a 3′ Overhang to a Blunt End

If there is no alternative restriction site, the 3′ overhang should be converted to a blunt end using the 3′→5′ exonuclease activity of Klenow DNA polymerase, as described below:

a. Set up a standard transcription *in vitro* reaction (Section B, below) minus the nucleotides and RNA polymerase.

b. Add Klenow DNA polymerase at a concentration of 5u/µg and incubate the reaction mixture for 15 minutes at 22°C.

c. Proceed with the transcription reaction by adding the nucleotide mix and RNA polymerase.

B. Synthesis of High Specific Activity Radiolabeled RNA Probes (20µl)

The protocols described below are a modification of the procedure described by Melton (1) for RNA synthesis *in vitro*. RNA transcripts may be radiolabeled with ^{32}P, ^{35}S or ^{3}H ribonucleotides, depending upon the specific application.

Certain applications, such as Southern and Northern blotting procedures, do not require the synthesis of full-length transcripts. Transcription mapping studies, on the other hand, require the synthesis of full-length labeled probes. The yield of full-length transcripts is reduced somewhat as the concentration of the limiting nucleotide falls below 12µM. Therefore, if you wish to omit unlabeled ribonucleotides from the reaction, increase the amount of label used in the reaction to maintain a final concentration of labeled ribonucleotide of 12-24µM. To keep the final reaction volume from exceeding 20µl, it may be necessary to add the labeled nucleotide to the reaction tube and dry it down before adding the remaining components. Table 6 lists the recommended specific radioactivities of labeled nucleoside triphosphates to be used for transcription reactions *in vitro*. Using an [α-^{32}P]CTP label and the conditions described below, RNA transcribed *in vitro* will typically have a specific radioactivity of 6-9 x 10^8 cpm/µg.

If RNA transcripts are to be used for probe hybridization, unincorporated nucleotides and template DNA should be removed to give lower backgrounds and optimum sensitivity (Sections E and F, pgs. 60-61).

III. RNA Transcription *in vitro*

(continued)

Table 6. Specific Activities of rNTPs Recommended for Transcription *in vitro*.

Nucleotide	Recommended Microcuries per Reaction	Specific Activity	Final Conc.
5′[α-^{32}P]CTP	50μCi	400Ci/mmol	6μM
5′[α-^{35}S]UTP	300μCi	1,300Ci/mmol	12μM
5,6 [^{3}H]UTP	25μCi	40Ci/mmol	31μM

Reagents to be Supplied by the User

- TE buffer (pg. 64)
- radiolabeled rNTPs
- RNA sample buffer (pg. 64)
- RNA loading buffer (pg. 64)

Standard Transcription Protocol

1. Add the following components at room temperature in the order listed:

transcription 5X buffer (pg. 64)	4μl
100mM DTT	2μl
rRNasin® ribonuclease inhibitor	20u
ATP, GTP, and UTP (2.5mM each) (prepared by mixing 1 volume H_2O with volume each of the 10mM ATP, GTP, and UTP stocks supplied)	4μl
100μM CTP	2.4μl
linearized template DNA (0.2-1.0mg/ml in water or TE buffer)	1μl
[α-^{32}P]CTP (50μCi at 10mCi/ml)	5μl
SP6, T3, or T7 RNA polymerase (at 15-20u/μl)	1μl
nuclease-free H_2O, if necessary	to final volume 20μl

Incubate for 60 minutes at 37-40°C.

Notes:

1. We do not recommend using radiolabeled ATP for generating RNA probes, since less label is generally incorporated using this nucleotide.
2. If you are preparing your own rNTP mixes, the nucleotide solution should be neutralized to pH 7.
3. The mixture should be kept at room temperature during the addition of each successive component, since DNA can precipitate in the presence of spermidine if kept at 4°C.

C. Synthesis of Large Amounts of RNA (100μl)

Using the conditions described below, yields of 5-10μg RNA/μg plasmid DNA can be obtained.

1. Add the following components at room temperature in the order listed:

transcription 5X buffer (pg. 64)	20μl
100mM DTT	10μl
rRNasin® ribonuclease inhibitor	100u
ATP, GTP, CTP and UTP (2.5mM each) (prepared by mixing equal volumes of the four 10mM rNTP stocks supplied)	20μl
linearized template DNA (2-5μg in water or TE buffer)	2μl
SP6, T3, or T7 RNA polymerase (at 15-20u/μl)	2μl
nuclease-free H_2O	to final volume100μl

Incubate for 60-120 minutes at 37-40°C.

Plasmid Cloning and Transcription *in vitro*

III. RNA Transcription *in vitro*

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D. Determination of Percent Incorporation and Probe Specific Activity

1. Estimate the dpm used in the synthesis reaction. If 30µCi was used, the dpm used was (30 x 2.2 x 10^6dpm/µCi) = 66 x 10^6dpm in 20µl, or 3.3 x 10^6dpm/µl.
2. Determine the percent incorporation using TCA precipitation. First, make a 1:10 dilution of the labeled probe in water. Spot 1µl of the 1:10 dilution onto duplicate glass fiber filters (e.g., Whatman GF/A) and let these air dry. Count these directly to determine the total cpm per microliter.
3. In duplicate tubes, add 1µl of the 1:10 dilution to 100µg of carrier nucleic acid (tRNA or herring sperm DNA) in a total volume of 100µl, mix, and then add 0.5ml of ice-cold 5% TCA and mix again. Leave on ice for at least 5 minutes.
4. Apply the samples to wet (5% TCA) GF/A filters under vacuum and wash twice with 5ml of ice-cold 5% TCA. Rinse the filters with 2ml of acetone and let them air dry.
5. Insert the dry filters into scintillation vials, add scintillation fluid, and count the samples. Calculate the average cpm from the duplicates (minus background):

   (TCA precipitated cpm/total cpm) x 100
   = percent incorporation
6. The specific activity of the probe may be expressed as the total incorporated cpm/total micrograms of RNA synthesized. The example below illustrates how an estimate may be made of the total incorporated cpm and the total micrograms of RNA synthesized.

   If 1µl of a 1:10 dilution was TCA precipitated, 10 x cpm precipitated = cpm/µl incorporated. In a 20µl reaction, the total cpm incorporated is 20 times this number.

   If 30µCi of labeled UTP at 400µCi/nmole were used, then 0.075nmoles of UTP were added to the reaction. If there was 100% incorporation and UTP represents 25% of the nucleotides in the probe, then 4 x 0.075 = 0.3nmoles of nucleotides were incorporated, and 0.3nmoles x 330ng/nmole = 99ng of RNA were synthesized (330ng/nmole = average molecular weight of a nucleotide). The percent incorporation calculated in step 5, above, allows calculation of the total RNA made: % incorporation x 99ng = total ng RNA made.

   For example, if 1µl of a 1:10 dilution contained 2.2 x 10^5cpm, then the total cpm incorporated were (10 x 20 x 2.2 x 10^5cpm) = 44 x 10^6 cpm. If 66 x 10^6cpm (30µCi) were available, then the percent incorporation was (44 x 10^6/66 x 10^6cpm) = 65%. The total RNA synthesized is then estimated to be (99ng x 0.65) = 64ng RNA. The specific activity of the probe is calculated to be (44 x 10^6cpm/0.064µg) = 4.7 x 10^8cpm/µg.

E. Removal of the DNA Template Following Transcription

The DNA template should be removed by digestion with DNase following the transcription reaction. Promega's RQ1 RNase-free preparation of DNase I has been tested for its ability to degrade DNA while maintaining the integrity of RNA.

After treatment with DNase, transcripts may be visualized by electrophoresis on denaturing gels. A phenol/chloroform extraction (Steps 3 and 4, below) should precede the addition of gel loading buffer to the sample. The aqueous phase can be mixed directly with loading buffer without the need for ethanol precipitation.

Reagents to be Supplied by the User

- RQ1 RNase-free DNase
- TE-saturated phenol/chloroform (pg. 64)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)

Protocol

After performing the *in vitro* transcription reaction:

1. Add RQ1 RNase-free DNase to a concentration of 1u/µg template DNA.
2. Incubate for 15 minutes at 37°C.
3. Extract with 1 volume of TE-saturated phenol/-chloroform (pg. 64). Vortex for 1 minute and centrifuge in a microcentrifuge (12,000 x g) for 5 minutes.

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III. RNA Transcription *in vitro*

(continued)

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 3. At this point, unincorporated nucleotides may be removed (Section F, below) or the RNA may be directly precipitated (Step 5).

   Note: To visualize transcripts by denaturing gel electrophoresis, add an aliquot of RNA (2-5µl) to 15-20µl of sample buffer. Add 2-5µl of loading buffer and heat the sample for 5-10 minutes at 65°-70°C prior to loading. Run the gel under normal conditions for analysis of DNA samples.

5. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate and 2.5 volumes of ethanol. Mix and place at –20°C for 30 minutes or on dry ice for 10 minutes. Centrifuge in a microcentrifuge for 5 minutes.

6. Carefully pour off the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and resuspend the RNA sample in 10-20µl of TE buffer or H_2O. Store at –70°C.

F. Precipitation of RNA and Removal of Unincorporated Nucleotides

Unincorporated nucleotides can be removed from the labeled probe by an ethanol precipitation step or by size-exclusion chromatography. These procedures are described below.

Reagents to be Supplied by the User

- 7.5M ammonium acetate
- ethanol (100% and 70%)
- TE buffer (pg. 64)

1. Ethanol Precipitation

This procedure removes the majority of unincorporated nucleotides and concentrates the RNA.

a. Add 0.5 volume of 7.5M ammonium acetate to the RNA sample and mix.

b. Add 2 volumes of ethanol, mix, and place at –20°C for 30 minutes.

c. Centrifuge in a microcentrifuge (12,000 x g) for 5 minutes. Carefully remove the supernatant

d. Resuspend the RNA pellet in 100µl of 1M ammonium acetate and mix.

e. Repeat the ethanol precipitation as described in Steps 1.b and 1.c, above.

f. The RNA sample may be stored at –70°C as an ethanol precipitate. Portions of the sample may be removed as an ethanol suspension, dried under vacuum, and resuspended in a volume of buffer appropriate to the intended application. Alternatively, the ethanol-precipitated pellet may be resuspended in 10-20µl of TE buffer and stored at –70°C.

2. Chromatography

The newly synthesized RNA may be most effectively separated from unincorporated nucleotides by size exclusion chromatography through a small Sephadex G-100 or G-50 column in 10mM Tris-HCl, pH 7.5, and 0.1% SDS.

a. Once the RNA has been separated from the unincorporated nucleotides, the sample should be ethanol precipitated as described in Steps 1.a-1.f. To ensure that the RNA is recovered successfully, carrier tRNA may be added to the reaction.

G. Capping RNA for Translation *in vitro*

RNA molecules synthesized *in vitro* are effective templates for translation. Krieg and Melton (15) have shown that SP6-derived mRNAs are translated as efficiently as native mRNAs in injected oocytes and in wheat germ extracts. These synthetic RNAs have also been successfully translated using the reticulocyte lysate system.

Two protocols for capping synthesized transcripts prior to translation are described below. The first protocol utilizes guanylyltransferase to transfer 5′ terminal cap structures to transcripts *in vitro*. Using the second protocol, the cap analogue is directly incorporated into the RNA during the transcription reaction to yield large amounts of capped RNA substrate.

Plasmid Cloning and Transcription *in vitro*

III. RNA Transcription *in vitro*

(continued)

Many RNA transcripts do not require a cap structure to be translated either in a rabbit reticulocyte lysate system or in a wheat germ extract system, while translation of other transcripts shows a direct dependence on the presence of the $m^7G(5')ppp(5')G$ cap at the 5′ end of RNA transcripts. It should also be noted that a low level of endogenous capping activity has been detected in wheat germ extract systems using *in vitro* transcribed RNA as the template.

The importance of a cap for synthesis of biologically active proteins has been reported to be particularly important for translation in *Xenopus* oocytes (18). Methylated capped RNA transcripts have also been shown to be spliced more efficiently than uncapped or unmethylated capped RNAs in an *in vitro* coupled transcription/splicing reaction (19).

Reagents to be Supplied by the User

- 1M Tris-HCl, pH 7.9
- 10mM $MgCl_2$
- 100mM KCl
- 1mM S-adenosyl-L-methionine
- guanylyltransferase
- TE-saturated phenol/chloroform (pg. 64)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)

1. Addition of a GpppG Cap to RNA Synthesized *in vitro*

a. Combine *in vitro* synthesized RNAs with guanylyltransferase in the following reaction mixture:

RNA transcript (in up to 13µl H_2O)	0.1-1.0µg
1M Tris-HCl, pH 7.9	1.5µl
10mM $MgCl_2$	3.75µl
100mM KCl	1.8µl
100mM DTT	0.75µl
1mg/ml acetylated BSA	3µl
RNasin® ribonuclease inhibitor (20-40u/µl)	1µl
1mM S-adenosyl-L-methionine	3µl
1mM GTP	1.2µl
guanylyltransferase (at 1-5u/µl)	1u/µg RNA
nuclease-free H_2O	to final volume 30µl

b. Incubate for 45 minutes at 37°C.

c. Extract with phenol/chloroform and then with chloroform alone. Precipitate with ethanol. (See Steps E.3-E.6, pg. 60.)

2. Synthesis *in vitro* of a Capped RNA Transcript

a. Synthesize RNA *in vitro* using the following reaction mix.

transcription 5X buffer	10µl
100mM DTT	5µl
1mg/ml acetylated BSA (optional)	5µl
RNasin® ribonuclease inhibitor	50u
nucleotides (add 2.5µl each of 10mM ATP, CTP and UTP plus 2.5µl of 1mM GTP)	10µl
5mM $m^7G(5')ppp(5')G$	5µl
linearized template DNA (1mg/ml in water or TE buffer)	5µl
SP6, T7 or T3 RNA polymerase	40u
nuclease-free H_2O	to final volume 50µl

b. Incubate at 37-40°C for 60 minutes.

Note: To increase the yield of RNA, add an additional 40 units of RNA polymerase and incubate for 60 minutes more.

c. Extract with phenol/chloroform and then with chloroform alone. Precipitate with ethanol. (See Steps E.3-E.6, pg. 60.)

III. RNA Transcription *in vitro* (continued)

H. Troubleshooting

Symptoms	Possible Causes	Comments
Low amounts of RNA synthesized utilizing standard transcription protocol.	Precipitation of the DNA template by the spermidine in the transcription 5X buffer.	Make sure the components of the reaction are assembled at room temperature and in the stated order.
	NaCl concentration high (>30mM).	Residual NaCl used to precipitate the template DNA may inhibit the RNA polymerase activity by as much as 50%. The template DNA may be desalted by column chromatography, reprecipitating the template in the presence of another salt, and washing the resulting pellet 1-2 times with 70% ethanol.
	RNase contamination.	The use of RNasin ribonuclease inhibitor is recommended for all transcription *in vitro* reactions. The transcription 5X buffer should be autoclaved. The other solutions used in the reaction (e.g., DTT and rNTPs) should be made up in water that has been treated with 0.1% diethyl pyrocarbonate. Individual transcription components may be purchased directly from Promega.
	Inactive RNA polymerase.	The activity of the individual RNA polymerase may be evaluated by transcription *in vitro* of the control template or supercoiled plasmid containing the appropriate RNA polymerase promoter.
Presence of incomplete transcripts.	Premature termination of RNA synthesis.	Increase the concentration of the limiting rNTP (probe synthesis only). Additional "cold" rNTP can be added to the reaction to increase the proportion of full-length transcripts. The improvement in yield of full-length product is gained at the expense of reducing the specific activity of the probe.
		Subclone the sequence of interest into a different vector in which transcription is initiated by a different RNA polymerase. Some sequences recognized as terminators by one RNA polymerase are not recognized as efficiently by another.
		Lower the temperature of incubation from 37°C to 30°C. This has been shown to increase the proportion of full-length transcripts in some cases (20).

(continued on next page)

Plasmid Cloning and Transcription *in vitro*

III. RNA Transcription *in vitro*
(continued)

H. Troubleshooting (continued from previous page)

Symptoms	Possible Causes	Comments
Presence of transcripts larger than expected	Transcription from the wrong strand of DNA.	If the DNA template has been linearized with a restriction enzyme that generates a protruding 3′ terminus, transcription results in the synthesis of significant amounts of long RNA molecules that are initiated at the terminus of the template (17). If it is impossible to avoid using a restriction enzyme of this type, the linear DNA should be "blunt ended" with Klenow before use in a transcription reaction (pg. 58).
	Nonlinearized plasmid is present in the sample.	Analyze the sample by gel electrophoresis. If undigested vector is noted, redigest with the appropriate restriction enzyme.

Composition of Solutions

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Transcription 5X buffer:

200mM	Tris-HCl, pH 7.5
30mM	$MgCl_2$
10mM	spermidine
50mM	NaCl

Nucleotide solutions: (made up separately)

10mM	ATP in H_2O, pH 7.0
10mM	GTP in H_2O, pH 7.0
10mM	CTP in H_2O, pH 7.0
10mM	UTP in H_2O, pH 7.0

RNA sample buffer:

10.0ml	deionized formamide
3.5ml	37% formaldehyde
2.0ml	MOPS buffer (0.2M MOPS, pH 7.0, 50mM sodium acetate, 5mM EDTA, pH 8.0)

Dispense into 500µl aliquots and store at –20°C in tightly sealed screw-cap tubes. These can be stored for up to 6 months. Do not freeze/thaw the buffer.

RNA loading buffer:

50%	glycerol
1mM	EDTA
0.4%	bromophenol blue
1µg/µl	ethidium bromide**

Make up 20ml using a very high grade glycerol such as that obtained from IBI (#15760). Most lower grades of glycerol will contain ribonuclease activity. The RNA loading buffer should be dispensed into 500µl aliquots and stored at –20°C.

*Buffer tablets available from Promega.

**Ethidium bromide can be added to the agarose gel (final concentration 0.5µg/ml) or added directly to the sample prior to loading.

IV. RNA Probe Hybridization Applications

An important application of the Riboprobe® system is the generation of radioactively labeled RNA hybridization probes. Single-stranded RNA probes offer a number of advantages over DNA probes for Southern and Northern blot hybridizations, including increased sensitivities and ease in preparation. Such probes have proven to be effective for analyzing single copy genes after relatively short exposure times. The greater thermal stability of RNA/DNA hybrids over DNA/DNA hybrids (21) is advantageous for Southern blots because it allows conditions of higher stringency to be used, thus yielding increased signal to noise ratios. The same advantage also applies when detecting RNA sequences (Northern blots) since RNA/RNA hybrids are more stable than DNA/RNA hybrids. Hybridization with RNA probes allows the background on blots to be further reduced by RNase A treatment to remove nonspecifically bound probes (see pg. 66, Note 2), a step which is not possible with DNA probes.

RNA probes may be used for plaque lift hybridization (5, pg. 67), colony hybridization (5,8), and hybridization *in situ* to tissue sections (22) and chromosome spreads (23,24).

In addition, RNA probes may be used for a variety of applications which employ solution hybridization. ^{32}P-labeled RNA probes can be used for the sensitive detection and quantitation of low abundance mRNA species (25). For this application, a complementary RNA probe transcribed *in vitro* is hybridized in solution with total cellular RNA, S1 nuclease is used to digest all unhybridized single-stranded RNA, and the remaining RNA:RNA hybrid is analyzed by polyacrylamide gel electrophoresis.

In a different application, S1 nuclease can be used to map the location of an RNA transcript hybridized to a larger, colinear DNA fragment (26,27,28). The RNA:DNA duplex region protected from S1 digestion is sized by electrophoresis on an alkaline agarose or polyacrylamide-urea gel. The RNA transcript can be mapped fairly precisely by performing this analysis with different, overlapping restriction fragments. RNA transcripts can be mapped on RNA as well as DNA templates (1,5,20). A variant of this technique has been used to accurately map sites of recombination and deletion in large viral RNA molecules (29). For these experiments, a combination of RNase A and RNase T1 was used to digest unhybridized single-stranded RNA.

Stringency of Hybridization

Hybridization and wash steps are generally carried out at 5-15°C below the T_m, the temperature at which 50% of hybrids are calculated to dissociate. The T_m is dependent on the solution conditions and the base composition of the probe, and may be calculated using the following equation (21):

$$T_m = 79.8°C + (18.5 \times \mathrm{Log}[Na^+]) + (58.4°C \times \%[G+C]) - 820/\#\mathrm{bp\ in\ duplex} - 0.5 \times \%\ \mathrm{formamide}$$

Formamide decreases the T_m of the hybrid and therefore allows lower incubation temperatures with RNA probes.

A. Southern and Northern Blot Hybridization Protocols

The DNA or RNA samples (for Southern or Northern blots, respectively) to be probed must first be immobilized on membranes composed of nitrocellulose, nylon, or PVDF (polyvinylidene difluoride). This may be accomplished by applying the sample directly to the membrane or by transferring the sample from a gel by vacuum, wicking, or electrophoresis (5). The same hybridization procedures may be used for samples applied to the membrane by any of these methods.

The most important factor for obtaining a high signal-to-noise ratio is the quality of the RNA probe. The probe specific activity (see pg. 60) should be greater than 10^8cpm/μg RNA. The probe should also be purified away from contaminating DNA, protein, and unincorporated nucleotides (see pgs. 60-61). Nonspecific binding of probe to the blot may be reduced by several means, including blocking the membrane with a protein-containing solution, addition of heterologous RNA, DNA and SDS to the hybridization buffer, and treatment with RNase A (see pg. 66, Note 2). Background can be reduced further by increasing the stringency of the hybridization and washing steps. A common practice is to perform washes at lower stringency and check the blot by autoradiography while it is still damp. If the background is too high, additional washes are then performed at higher stringency.

Plasmid Cloning and Transcription *in vitro*

IV. RNA Probe Hybridization Applications

(continued)

Since a variety of RNA probe hybridization protocols appear to be successful for different users, we do not recommend one specific protocol over others. Included below is an example of a protocol for hybridization to nitrocellulose or nylon membranes. References for several other hybridization protocols are supplied on pg. 73.

Reagents to be Supplied by the User

Instructions for preparing these buffers are provided on pg. 69.

For nitrocellulose membranes:
- hybridization buffer A
- 1X SSC

For nylon membranes:
- hybridization buffer B
- 2X SSC
- PSE buffer
- PES buffer

Optional:
- RNase A

Hybridization Protocol

This protocol is designed for hybridizations using 82mm circular membranes. Volumes should be adjusted accordingly for different membrane sizes.

1. Place the membrane(s) to be hybridized with each probe in a heat sealable plastic bag. Heat seal three of the four sides of each bag within 1cm of the edge of the membrane(s).
2. Add 5-10ml of the desired hybridization buffer per membrane (see Tables 7 and 8) to wet the membrane(s).
3. Seal the fourth side of each bag, leaving room to cut and reseal the bag after the prehybridization step.
4. Prehybridize by incubating the sealed bags as indicated in Table 7 or 8.
5. Cut off one corner of each bag, squeeze out the buffer, and add 5ml of fresh hybridization buffer per membrane.
6. Add labeled probe to a concentration of 1 x 10^6 incorporated cpm/ml of buffer. Heat seal the pouches, including as few bubbles as possible.
7. Hybridize by incubating the sealed bags as indicated in Table 7 or 8.
8. Remove the membrane(s) from each bag and wash at room temperature (22°C) with gentle shaking, as described in Table 7 or 8.
9. Wash at 65°C, as described in Table 7 or 8. Shaking is not necessary. If desired, RNase A may be used at this step to reduce background resulting from nonspecifically bound probe (see Note 2, below).
10. Blot each membrane on filter paper to remove excess buffer. **Note:** The membrane should not be completely dried if additional washes are to be performed or if the membrane is to be stripped and reprobed. The probe will remain permanently bound to the membrane if the blot is dried.
11. Mount each membrane on filter paper with the probe side up. Mark the edges of the membrane with radioactive ink, if desired, to help orient the autoradiogram, and cover the membrane with plastic wrap.
12. Expose the membrane(s) to X-ray film for 2 hours to overnight, depending on the desired intensity and level of background. The use of a calcium tungstate intensifying screen (arranged as sample:film:screen) enhances the sensitivity of the film 4- to 5-fold (5,30).

Notes:

1. For screening plaque lifts, mark positives by correctly orienting the autoradiogram over the membrane and pushing a needle through the film and the membrane. Then lay the membrane over the original plate by matching the needle holes made when doing the plaque lift, and identify the positive plaques. Plug the positives and plaque purify as described in reference 5.
2. RNase A treatment may be used to remove non-specifically bound probe. Rinse the membrane for 3 times, 5 minutes each, in 2X SSC and then incubate it at room temperature (22°C) for 15 minutes in 2X SSC containing 1 µg/ml RNase A. Rinse the filter for 30 minutes at 50°C in 0.1X SSC, 0.1% SDS.

IV. RNA Probe Hybridization Applications

(continued)

Table 7. Blot Hybridization Conditions for Nitrocellulose Membranes.

Step	Buffer Composition	Incubation Conditions
Prehybridization	Hybridization Buffer A (pg. 69)	50°C, 15 minutes (for Southern blots) or 55-60°C, 15 minutes (for Northern blots)
Hybridization	Hybridization Buffer A	50°C, 12-19 hours (for Southern blots) or 55-60°C, 12-19 hours (for Northern blots)
Washes	1. 1X SSC, 0.1% SDS	22°C, 30 minutes (with gentle shaking)
	2. 1X SSC, 0.1% SDS	22°C, 30 minutes (with gentle shaking)
	3. 0.1X SSC, 0.1% SDS	65°C, 30 minutes
	4. 0.1X SSC, 0.1% SDS	65°C, 30 minutes

Table 8. Blot Hybridization Conditions for Nylon Membranes.

Step	Buffer Composition	Incubation Conditions
Prehybridization	Hybridization Buffer B (pg. 69)	42°C, 15 minutes (for Southern blots) or 55-60°C, 15 minutes (for Northern blots)
Hybridization	Hybridization Buffer B	42°C, 12-19 hours (for Southern blots) or 55-60°C, 12-19 hours (for Northern blots)
Washes	1. 2X SSC (pg. 69)	22°C, 5 minutes (with gentle shaking)
	2. PSE Buffer (pg. 69)	65°C, 20-60 minutes
	3. PSE Buffer	65°C, 20-60 minutes
	4. PES Buffer (pg. 69)	65°C, 20 minutes
	5. PES Buffer	65°C, 20 minutes

B. Plaque Lift Hybridizations (5)

Reagents to be Supplied by the User

- 1.5M NaCl, 0.5M NaOH
- neutralizing solution (pg. 69)

Protocol

1. When plaques reach the desired size (usually 1-2mm), cool the plates for 15-30 minutes at 4°C to reduce the likelihood of the top agarose sticking to the membrane.
2. Hold a dry nitrocellulose or nylon membrane by the edges (with gloved fingers), bend it slightly to produce a bow in the center, and carefully lay the membrane on top of the plate, center first. Let the sides of the membrane drop onto the plate as the membrane wets. This procedure helps to avoid trapping bubbles. Leave the membrane on the plate for one minute after it is completely wetted.
3. Mark the filter in at least three asymmetric locations around the edges by stabbing through it with a needle into the agar below. You may mark the pinholes in the membrane with waterproof ink to make them easier to see.

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IV. RNA Probe Hybridization Applications

(continued)

4. Using a blunt forceps, carefully peel the membrane away from the top agarose surface. Immerse the membrane, DNA side up, in a shallow tray containing 1.5M NaCl, 0.5M NaOH for 60 seconds. The alkaline pH of this solution denatures double-stranded DNA bound to the membrane. Duplicate filters can be made from the same plate using the same procedure. For each replica, however, the incubation time on the plate should be increased by 30 seconds.

5. Transfer the membrane to another tray containing neutralizing solution (pg. 69) for 5 minutes.

6. Place the membrane, DNA side up, on filter paper (e.g., Whatman 3MM) and allow it to dry.

7. Place the membrane between sheets of filter paper, wrap it in aluminum foil, and bake it in a vacuum oven at 80°C for 15-30 minutes. This step irreversibly binds the DNA to the membrane. The use of a vacuum oven is not required for nylon membranes.

8. Store the baked membrane wrapped in plastic at 4°C.

C. Transcription Mapping

^{32}P-labeled RNA probes can be used for the detection, quantitation, and structural analysis of specific RNAs in solution. In the RNase mapping protocol described below (1,5,14), ^{32}P-"antisense" RNA synthesized using the Riboprobe system is hybridized in solution to the RNA being studied. Treatment with RNase A and RNase T1 results in digestion of the unhybridized single-stranded ^{32}P-RNA probe. Double-stranded ^{32}P-RNA:RNA duplexes are resistant to ribonuclease and are detected by subsequent gel electrophoresis.

The RNA probe to be used should be labeled to a high specific activity (≥10^8cpm/µg), free of template DNA (see pg. 60), and full-length. If shorter RNA species are present, full-length transcripts may be isolated directly from an agarose or acrylamide gel. Using a highly labeled probe (>10^9cpm/µg), 2-20µg of test RNA should allow detection of mRNA species present in 1-5 copies per cell. If greater sensitivity is needed, up to 150µg of test RNA may be used in this procedure.

Reagents to be Supplied by the User

Instructions for preparing these reagents are provided on pgs. 69-70.

- 7.5M ammonium acetate
- ethanol (100% and 70%)
- 80% formamide hybridization buffer
- RNase digestion solution
- 10% SDS
- 10mg/ml proteinase K
- TE saturated phenol/chloroform
- formamide loading buffer

Protocol

1. Ethanol precipitate 2-150µg of the test RNA as described on pg. 61, Steps E.5-E.6. Remove the ethanol and allow the pellet to dry at room temperature.

2. Add a small molar excess of the appropriate ^{32}P-labeled RNA probe (see Note 1, below) to 30µl of 80% formamide hybridization buffer (pg. 69) and use this solution to resuspend the test RNA pellet. Pipet the sample up and down several times to thoroughly resuspend the pellet.

3. Heat the sample to 85°C for 5 minutes to denature the RNAs and then incubate the sample for 6 hours to overnight at the chosen annealing temperature (see Note 2, below).

4. Cool the sample to room temperature, add 300µl of RNase digestion solution (pg. 69), and incubate at 30°C for 60 minutes (see Note 3, below).

5. To terminate the RNase digestion, add 20µl of 10% SDS and 10µl of freshly prepared proteinase K (10mg/ml in H_2O). Incubate the sample at 37°C for 30 minutes.

6. Add 400µl of TE-saturated phenol/chloroform (pg. 70), vortex for 1 minute, and centrifuge at 12,000 x g for 5 minutes.

7. Transfer the upper, aqueous phase to a fresh tube and add 1ml of ice-cold ethanol. Mix and allow the RNA to precipitate at –20°C for 30 minutes.

IV. RNA Probe Hybridization Applications

(continued)

8. Centrifuge at 12,000 x g for 5 minutes. Carefully rinse the pellet in 10µl of formamide loading buffer (pg. 70), heat to 95°C for 5 minutes, and chill in an ice bath.

9. Analyze the labeled RNA by electrophoresis on a denaturing polyacrylamide/8M urea gel followed by autoradiography (5).

Notes:

1. The optimal amount of probe should be determined empirically using a series of reactions containing varying ratios of probe and test RNA. When comparing different test RNA preparations, the total amount of test RNA in each digestion should be equalized by adding carrier tRNA as needed.

2. The hybridization temperature should be empirically determined, and is dependent upon parameters such as the G-C content of the test RNA and the length of the RNA:RNA duplex. For most ^{32}P-RNA:RNA duplexes, however, 45°C is a satisfactory hybridization temperature.

3. If the signal to noise ratio is too low, the time and temperature of the RNase digestion should be determined empirically.

Composition of Solutions

1X SSC*:

0.15M	NaCl
0.015M	sodium citrate, pH 7.0

20X SSC:

3M	NaCl
0.3M	sodium citrate, pH 7.0

Hybridization buffer A:

50%	formamide, deionized
6X	SSC
1%	SDS
0.1%	Tween 20
100µg/ml	tRNA

Hybridization buffer B:

50%	formamide, deionized
0.25M	$NaPO_4$, pH 7.2
0.25M	NaCl
1mM	EDTA
7%	SDS
5-10%	polyethylene glycol, M.W. 8,000 (optional)

Solid SDS and PEG are added after the rest of the components have been mixed and warmed to 42°C. Hybridization is usually at 42°C for DNA targets on nylon membranes.

PSE buffer:

0.25M	sodium phosphate, pH 7.2
2%	SDS
1mM	EDTA

PES buffer:

0.04M	sodium phosphate, pH 7.2
1%	SDS
1mM	EDTA

Neutralizing solution:

0.5M	Tris-HCl, pH 8.0
1.5M	NaCl

80% Formamide hybridization buffer:

80%	formamide, deionized
40mM	PIPES, pH 6.7
0.4M	NaCl
1mM	EDTA

RNase digestion solution:

10mM	Tris-HCl, pH 7.5
5mM	EDTA
300mM	NaCl
2µg/ml	RNase T1
40µg/ml	RNase A

Store these RNases in stock solutions at –20°C: RNase T1, 100µg/ml; RNase A, 10mg/ml.

*Buffer tablets available from Promega.

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Plasmid Cloning and Transcription *in vitro*

IV. RNA Probe Hybridization Applications

(continued)

Formamide loading buffer:

80%	formamide, deionized
10mM	EDTA
1mg/ml	xylene cyanol FF
1mg/ml	bromophenol blue

TE-saturated phenol/chloroform:
Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

V. High Level Expression of Fusion Proteins with pGEMEX™ Vectors

Clone the DNA fragment to be expressed into the pGEMEX™-1 or pGEMEX™-2 vector using the procedures described in Sections II.A and II.B (pgs. 51-52). The reading frame of the pGEMEX-1 vector at the *Eco*R I site is identical to that of λgt11, and the reading frame of the pGEMEX-2 vector at the *Sfi* I site is identical to that of the λgt11 *Sfi-Not* directional cloning vector. Multiple cloning site sequences for these vectors are provided on pgs. 362-364.

A. Expression of T7 Gene 10 Fusion Protein

1. Transform the recombinant pGEMEX plasmid into competent cells of the JM109(DE3) bacterial strain (see Note 1). Procedures for competent cell preparation and transformation are provided in Section II.C (pg. 52).

2. Pick recombinant colonies and grow them overnight at 37°C in 50ml of LB medium (pg. 57) containing 100µg/ml ampicillin. Blue/white screening is not possible with the pGEMEX vectors.

3. The next day remove 1-2ml of the culture and inoculate it into 50ml of fresh LB medium containing 100µg/ml ampicillin.

4. Allow the culture to grow to an OD_{600} of 0.2-0.5 (3-5 hours). Add IPTG to a final concentration of 0.5mM.

5. Incubate the culture for an additional 3-4 hours. Incubation overnight can further increase the yield of the expressed fusion protein.

B. Fusion Protein Solubilization for Gel and Western Blot Analysis

The T7 gene 10 leader peptide often causes the fusion protein to be insoluble unless cell lysis is performed in a high salt buffer. We recommend the following procedure.

1. Remove 100-200µl of the culture and pellet the bacterial cells in a microcentrifuge.

2. Resuspend the bacterial pellet in 100µl of 50mM Tris-HCl, pH 7.5, 2mM EDTA, 0.1M NaCl. The NaCl helps to solubilize the fusion protein (see Note 2).

3. Lyse the cells by adding lysozyme to a final concentration of 100µg/ml. Incubate at 30°C for 15 minutes.

4. Spin a portion of the sample in a microcentrifuge for 2 minutes. This pellets insoluble protein.

5. Analyze the protein content of the total lysate, the supernatant (which should contain the solubilized fusion protein), and the resuspended pellet by SDS gel electrophoresis (pg. 170).

6. The gel may be stained with Coomassie blue or the separated proteins may be transferred to a nitrocellulose membrane and probed with a specific antibody to the protein of interest (31).

Notes:

1. All initial cloning should be performed in bacterial strains other than JM109(DE3). The bacterial strain JM109(DE3) tends to "lose" plasmids, especially if the expressed recombinant protein is toxic.

V. High Level Expression of Fusion Proteins with pGEMEX™ Vectors

(continued)

2. If maintaining the solubility of the fusion protein is not important, resuspend the cells in 50µl of TE (pg. 64) buffer and rapidly lyse them by adding 50µl of SDS sample buffer (pg. 172) and boiling for 2-3 minutes. Load the sample directly on an SDS polyacrylamide gel.
3. Expression of certain toxic genes can kill at saturation, although little effect is observed on cells in logarithmic growth.
4. The T7 terminator present in the pGEMEX vectors is approximately 70-80% efficient. If the target DNA does not contain an internal termination sequence, transcription past the T7 terminator may result in fusion proteins larger than expected.
5. Alternative approaches (e.g., urea, Triton X-100) for solubilizing fusion proteins can be found in reference 32.

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Transcription *in vitro* Systems Technical Manual

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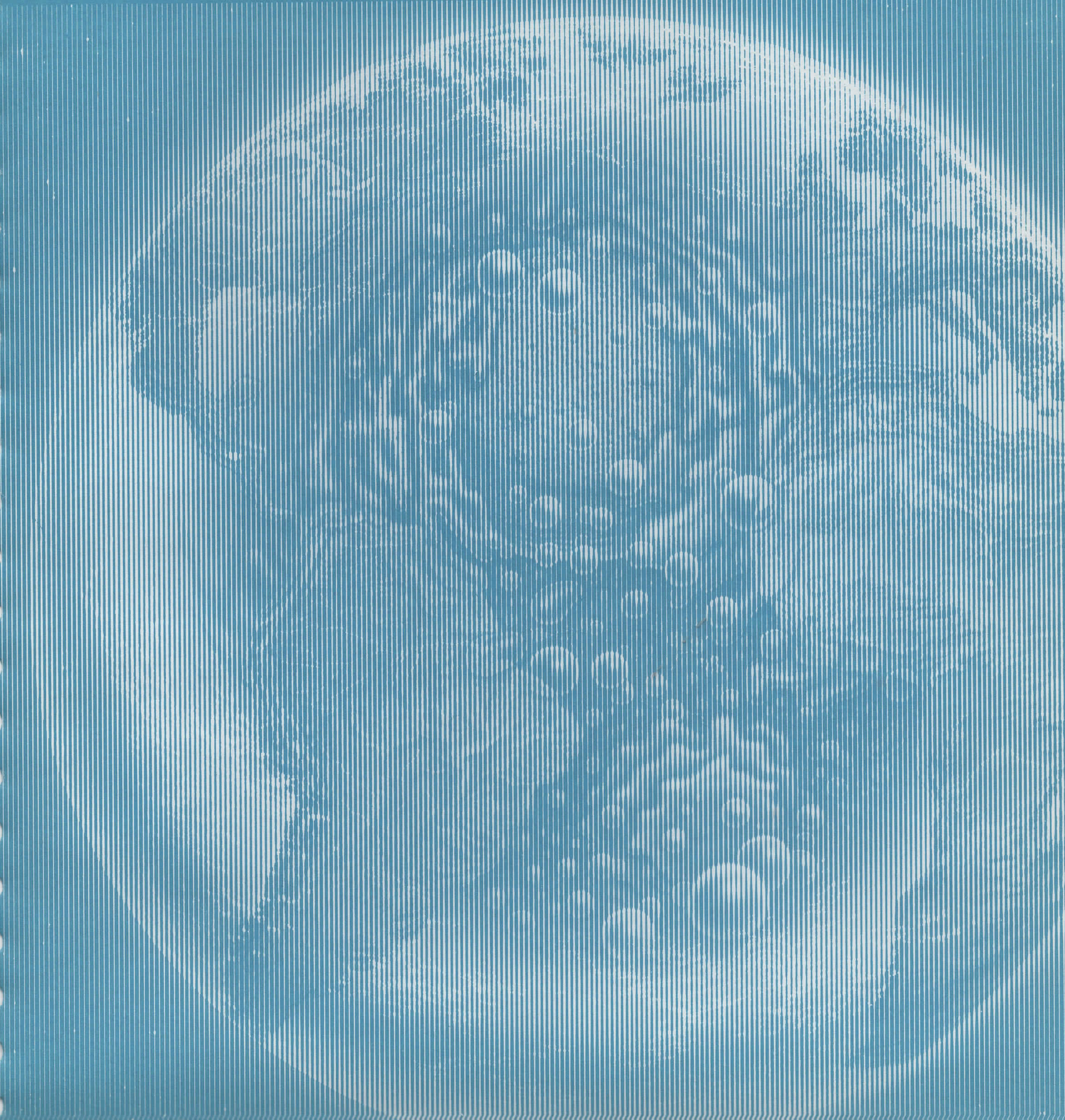
PROTOCOLS AND APPLICATIONS GUIDE



Nucleic Acid Sequencing and Mutagenesis

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Nucleic Acid Sequencing and Mutagenesis

I. General Considerations for Dideoxy Sequencing

The sequence of a deoxyribonucleic acid molecule can be determined by chemical (1) or enzymatic (2) methods. The enzymatic method of sequencing is based on the ability of a DNA polymerase to extend a primer, hybridized to the template to be sequenced, until a chain-terminating nucleotide is incorporated. Each sequence determination is carried out as a set of four separate reactions, each of which contains all four deoxyribonucleoside triphosphates (dNTP) supplemented with a limiting amount of one dideoxyribonucleoside triphosphate (ddNTP). Because the ddNTP lacks the necessary 3′-OH group required for chain elongation, the growing oligonucleotide is terminated selectively at G, A, T, or C, depending on the respective dideoxy analog in the reaction. The relative concentrations of dNTPs and ddNTPs can be adjusted to give a nested set of terminated chains from several hundred to a few thousand bases in length. The resulting fragments, each with a common origin but ending in a different nucleotide, are separated according to size by high resolution denaturing gel electrophoresis.

A. Sequencing Templates

A variety of nucleic acid templates are suitable for sequencing. DNA inserts cloned into M13 or phagemid vectors (such as the pGEM®-Zf series) can be expressed as single-stranded DNA (ssDNA) molecules ideally suited for sequencing. However, recombinant plasmids also can be sequenced directly without additional subcloning or single-stranded template preparation steps. Supercoiled plasmid DNA can be alkali denatured, neutralized, annealed to a primer, and sequenced by standard dideoxy methods (3). Direct sequence analysis of DNA cloned into lambda vectors is also possible when using *Taq* DNA polymerase (Sections II and III). Finally, RNA templates may be directly sequenced using reverse transcriptase (Section V).

Sequence data from large DNA inserts (longer than 500-600 bases) may be obtained by using the Erase-a-Base® system. A nested set of unidirectional deletions is generated, effectively moving internal regions of the DNA template adjacent to the sequencing primer binding site so that the entire insert can be sequenced with the same primer (Figure 1, pg. 90).

The bacteriophage SP6 and T7 RNA polymerase promoters present on pGEM® plasmids serve as convenient and specific priming sites for sequencing reactions. Both ends of an insert can be quickly sequenced since the promoters flank the multiple cloning sites into which foreign DNA is inserted. Sequencing primers are available for several common cloning vectors, including pUC, M13 and lambda vectors (Section XII, pg. 119). Promega also supplies custom oligonucleotides for researchers who choose to design their own sequencing primers.

B. Sequencing Enzymes

Four DNA polymerases are commonly used for sequencing nucleic acids: Klenow fragment, AMV reverse transcriptase (RT), *Thermus aquaticus*

Table 1. Comparison of Sequencing Enzyme Properties.

	Processivity	Rate of Incorporation	Exonuclease Activity	Templates	Sequencing Temperature
Klenow Fragment	low	10-12dNTP/sec	3′→ 5′	DNA	37°C
AMV Reverse Transcriptase	moderate	4dNTP/sec	none	DNA & RNA	42°C
Taq DNA Polymerase	moderate	>60dNTP/sec	weak 3′→ 5′, inactivated in Promega's sequencing grade enzyme*	DNA	70° - 80°C
Modified T7 DNA Polymerase	high	>300dNTP/sec	3′→ 5′ inactivated	DNA	37°C

*The weak 5′→ 3′ exonuclease activity of *Taq* DNA polymerase is not detectable in standard exonuclease assays, but the presence of this activity can be inferred from the behavior of the enzyme in other reactions (7).

I. General Considerations for Dideoxy Sequencing

(continued)

(*Taq*) DNA polymerase, and modified T7 DNA polymerase. Klenow fragment and RT have been used for many years for dideoxy sequencing and are well-characterized. The *Taq* and T7 DNA polymerases, introduced more recently, offer certain advantages for sequencing DNA templates.

Klenow and RT differ in their ability to read accurately through certain sequences. While with the majority of sequences (>80%) either enzyme generates clean sequence ladders, with certain template regions one enzyme may be preferable for unambiguous sequence determination. Examples of sequences which are read differently by the two enzymes (2,4,5,6) are given below:

a. Regions rich in dA:dT residues are read more accurately by Klenow than by RT.

b. Regions with long dG or dC tails are read less ambiguously with RT than with Klenow.

c. Where there are multiple dC residues in a row, RT reads them all with even intensity. In contrast, with Klenow the first dC is consistently fainter than the following dCs and may sometimes be missed in normal autoradiography exposures.

Since these two enzymes tend to have difficulties on different types of sequences, in most cases an unambiguous sequence can be determined using both enzymes. For this reason, Promega provides both Klenow and RT in the K/RT® sequencing systems.

The *Taq* (7) and modified T7 (8) DNA polymerases offer significant advantages over Klenow and RT for DNA sequencing applications (Table 1). Both have a high rate of incorporation, good processivity, and lack 3′→ 5′ exonuclease activity. As a result, sequence data obtained with these enzymes exhibits a consistent band intensity, low background, and a high degree of accuracy (7,8). The higher reaction temperature of the *Taq* polymerase sequencing reaction (70-80°C), however, decreases the secondary structure of DNA templates and thus permits polymerization through highly structured regions. The higher temperature also increases the stringency of primer hybridization. This enzyme is useful for sequencing a wide variety of templates such as amplified DNA, GC-rich templates and large double-stranded (dsDNA) DNA templates such as lambda.

Promega's *Taq* Track® sequencing system is optimized to produce readable sequence data from 10 to 500 bases. Two configurations of nucleotide mixes are available: a regular nucleotide mix for routine sequencing and a deaza nucleotide mix configuration that substitutes 7-deaza dGTP for dGTP to resolve band compressions. Promega has recently developed a specially modified sequencing grade of *Taq* DNA polymerase* which exhibits essentially no 5′→ 3′ exonuclease activity. This lack of exonuclease activity makes the modified polymerase the preferred enzyme for sequencing. The presence of 5′→ 3′ exonuclease activity in DNA sequencing can result in degradation of the primer from the 5′ end such that the starting nucleotide in the reaction is variable. When sequencing dsDNA with an internally incorporated label, 5′→ 3′ exonuclease activity can result in a high background of artifactual bands, making interpretation of DNA sequencing data difficult if not impossible. Use of the modified *Taq* DNA polymerase allows the distinct and unambiguous electrophoretic separation of DNA fragments and thereby generates reliable sequence data.

*Patent Pending

C. Labeling Methods

Incorporating a radiolabel somewhere in the oligonucleotide chain permits the visualization of the sequencing products by autoradiography. Three basic radiolabeling protocols have been utilized to detect the reaction products. The original method developed by Sanger (2) combines the labeling and the primer extension/chain termination portions of the reaction into one step by lowering the concentration of one of the four dNTPs and adding the same radioactively labeled dNTP. This labeling method is most commonly used for sequencing procedures employing Klenow fragment and AMV reverse transcriptase (RT). A second labeling method, developed by Tabor and Richardson (8), separates the sequencing reaction into a labeling step and an extension/termination step. In the first step, the primer is extended a short distance using limiting concentrations of the dNTPs and a single radiolabeled dNTP. In the second step, the "extended primers" are further extended in the presence of both dd- and dNTPs. A third means of radiolabeling is to directly attach a label to the end of the primer (9,10). The oligonucleotide

I. General Considerations for Dideoxy Sequencing

(continued)

is 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. The subsequent extension/termination reaction is not limiting for one of the dNTPs. These latter two labeling methods can be used for sequencing with *Taq* and T7 DNA polymerase.

The use of an end-labeled primer affords certain advantages in sequencing. Since label is only incorporated into the specific sequencing primer, chains extended from other small DNA fragments do not contribute to background on the sequencing gel. A significant application of the end-labeled primer method is the sequencing of large double-stranded templates such as lambda. The single radiolabel present per molecule results in an essentially uniform band intensity throughout the sequencing ladder. Therefore, 500 bases of ^{32}P labeled sequence can be read with a single exposure of X-ray film. Finally, degradation of the sequencing products by radiolysis is not a problem when using ^{32}P-end labeled sequencing primers. The radiolysis that does occur simply results in unlabeled fragments. The end-labeled primers and their extension products may be stored frozen at –20°C for as long as a month and still generate clear sequence data.

A disadvantage of using an end-labeled primer is that, with ^{35}S as the label, very long exposure times are required (72-96 hours). This problem can be overcome by using the two-step labeling method described above. This protocol gives higher label incorporation, making overnight exposures possible when sequencing with ^{35}S-labeled nucleotides.

II. *Taq* DNA Polymerase Sequencing Using an End-Labeled Primer

The end-labeled primer sequencing protocol can be divided into three steps; 1) end-labeling the sequencing primer, 2) annealing the labeled oligonucleotide and template, and 3) the extension/termination reaction. Each step requires 10-15 minutes, not including setup time. The TaqTrack® system has been optimized to produce readable sequence data from 10 to 500 bases with M13mp18 ssDNA as the template and a reaction temperature of 70°C. Depending on the quality of the DNA preparation, approximately 200-400 bases of readable sequence can be obtained from a double-stranded DNA (dsDNA) template. If an especially difficult secondary structure is encountered, increasing the reaction temperature to 80 or 85°C will facilitate polymerization through such structures, although the length of read may decrease. Sequencing grade *Taq* DNA polymerase is stable to 95°C.

Reagents to be Supplied by the User

- [γ-^{32}P]ATP, >3,000Ci/mmol

A. Radioisotope Considerations

The end-labeled primer sequencing protocol is optimized for use with [γ-^{32}P]ATP having a specific activity of 3,000Ci/mmol or greater. Higher specific activities can be used with equivalent results, however it may be important to utilize a carrier-free label (99% pure) to obtain optimal results. Depending on the specific activity of the ^{32}P used, autoradiograms can be obtained in 2-12 hours with an intensifying screen. An increase in resolution can be achieved by exposure without an intensifying screen for 12-72 hours. Because of the long exposure times that would be required (72-96 hours) when using a [γ-^{35}S] end-labeled primer, the two-step extension/labeling protocol using [α-^{35}S]dATP (Section III, pg. 83) is recommended for sequencing with an ^{35}S label. A specific activity greater than 1,000Ci/mmol is recommended for [α-^{35}S]dATP.

B. Primer Radiolabeling Reaction

The following protocol is designed to label enough primer for 5 sets of double-stranded or 10 sets of single-stranded sequencing reactions, and it can be scaled proportionately according to the number of reactions to be performed. If the volumes of the radiolabel or primer are in excess, they can be concentrated by drying in a vacuum desiccator and redissolving in the appropriate volume of buffer. The amount of ^{32}P in the reaction should be doubled if the isotope has decayed by one half-life (approximately 14 days).

1. In a microcentrifuge tube combine the following:

primer (Table 2)	10pmol
γ-labeled ATP (Table 3)	10pmol
polynucleotide kinase 10X buffer (pg. 81)	1µl
T4 polynucleotide kinase (5-10u/µl)	5u
sterile H_2O	to final volume 10µl

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II. *Taq* DNA Polymerase Sequencing Using an End-Labeled Primer

(continued)

2. Incubate at 37°C for 10 minutes and then inactivate the kinase at 90°C for 2 minutes.
3. Briefly spin in a microcentrifuge to bring down any condensation. The end-labeled primers (and their extension products) may be stored frozen at –20°C for as long as a month and still generate clear sequence data. The primer may be used directly without further purification.

Note: Different templates or sequencing strategies require various lengths of oligonucleotide primer. Table 2 provides a guide for determining the number of nanograms (ng) that is equivalent to 10pmol of primer molecules. Table 3 shows the corresponding amounts of gamma labeled ATP to use in the kinase reaction.

C. Annealing the Template and Primer

1. Annealing Single-Stranded DNA Template and Primer

Single-stranded DNA suitable for sequencing can be prepared from M13 or phagemid vectors (Section IX, pg. 109). The recommended amount of single-stranded DNA (ssDNA) template to use per set of sequencing reactions is 0.8pmol,

Table 2. Amount of Sequencing Primer (ng) Needed to Equal 10pmol.

Primer Length	ng of Primer Equal to 10pmol
15mer	50ng
16mer	53ng
17mer	56ng
18mer	59ng
19mer	63ng
20mer	66ng
24mer	80ng

Table 3. Amount of Radiolabel Needed to Equal 10pmol.

[γ-^{32}P]ATP:	3.0μl of 3,000Ci/mmol, 10μCi/μl
	5.0μl of 5,000Ci/mmol, 10μCi/μl
	0.5μl of 6,000Ci/mmol, 135μCi/μl

or approximately 2μg of an 8kb M13 template. Anneal the radiolabeled primer with the ssDNA template in an approximately 1:1 molar ratio. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

ssDNA (approx. 2μg of an M13 template)	0.8pmol
Taq DNA polymerase 5X buffer (pg. 81)	5.0μl
labeled primer (1pmol)	1.0μl
sterile H_2O	to final volume 25μl

Incubate at 37°C for 10 minutes. While this reaction is incubating, prepare the extension/termination reaction tubes as described on pg. 81, Steps 1 and 2.

2. Annealing Double-Stranded Plasmid Template and Primer

The recommended amount of double-stranded plasmid template to use per set of sequencing reactions is 1.6pmol, which represents approximately 4μg of a 3-5kb plasmid vector. Prior to annealing, alkali denature and precipitate the template (pg. 107, Section VIII.C). The radio-labeled primer is annealed with the dsDNA plasmid template in an approximately 1:1 molar ratio. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

denatured plasmid dsDNA (approx. 4μg of a 3-5kb template)	1.6pmol
Taq DNA polymerase 5X buffer (pg. 81)	5.0μl
labeled primer (2pmol)	2.0μl
sterile H_2O	to final volume 25μl

Incubate at 37°C for 10 minutes. While the annealing reaction is incubating, prepare the extension/termination reaction tubes as described on pg. 81, Steps 1 and 2.

II. *Taq* DNA Polymerase Sequencing Using an End-Labeled Primer

(continued)

3. Annealing Lambda Template and Primer

The recommended amount of double-stranded lambda template to use per set of sequencing reactions is 0.4pmol, which represents 10µg of an approximately 42kb lambda vector. Prior to annealing, alkali denature and precipitate the template (Section VIII.D, pg. 108). The radiolabeled primer is annealed with the dsDNA lambda template in an approximately 3:1 molar ratio. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

denatured lambda dsDNA (approx. 10µg of a 42kb template)	0.4pmol
Taq DNA polymerase 5X buffer (below)	5.0µl
labeled primer (1pmol)	1.0µl
sterile H_2O	to final volume 25µl

Incubate at 37°C for 10 minutes. While the annealing reaction is incubating, prepare the extension/termination reaction tubes as described below, Steps 1 and 2.

D. Extension/Termination Reactions

1. For each set of sequencing reactions, label four microcentrifuge tubes (G, A, T, and C) and add 1µl of the appropriate d/ddNTP mix to each tube. Cap the tubes and store on ice or at 4°C until needed.
2. Add 1.6µl of enzyme (sequencing grade *Taq* DNA polymerase at 2.5u/µl) to the annealed primer/template mix (from Step C.1, C.2, or C.3 above).
3. Add 6µl of the enzyme/primer/template mix (from Step D.3) to each of the four tubes containing the d/ddNTP mixes. Mix briefly by pipetting up and down. A brief spin may be needed to ensure that no liquid is left on the tube walls.
4. Incubate at 70°C for 15 minutes.
5. After 15 minutes, add 4µl of stop solution to each tube and set at room temperature.
6. Heat the reactions to ≥70°C for 2-5 minutes before loading the sequencing gel. Load 2.5-3.0µl of each reaction on the gel.

Notes:

1. Reactions can be stored overnight at –20°C or –70°C.
2. The calculated T_m for a sequencing primer is often less than 70°C, the temperature of the sequencing reation. However, this descrepancy does not result in interference with primer annealing and extension. A likely explanation for this observation is that primer/template complexes annealed at 37°C are rapidly stabilized by extension with *Taq* DNA polymerase as the reaction warms to 70°C.

Composition of Solutions

Polynucleotide kinase (PNK) 10X buffer (supplied):

500mM	Tris-HCl, pH 7.5
100mM	$MgCl_2$
50mM	DTT
1.0mM	spermidine

***Taq* DNA polymerase 5X buffer (supplied):**

250mM	Tris-HCl, pH 9.0 at 25°C
50mM	$MgCl_2$

Stop solution (supplied):

10mM	NaOH
95%	formamide
0.05%	bromophenol blue
0.05%	xylene cyanol

Nucleic Acid Sequencing and Mutagenesis

II. *Taq* DNA Polymerase Sequencing Using an End-Labeled Primer

(continued)

Table 4. Regular Nucleotide Mix Formulations for *Taq* DNA Polymerase.

Component	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	50µM	—	—	—
ddATP	—	350µM	—	—
ddTTP	—	—	300µM	—
ddCTP	—	—	—	160µM
dGTP	25µM	250µM	250µM	250µM
dATP	250µM	25µM	250µM	250µM
dTTP	250µM	250µM	25µM	250µM
dCTP	250µM	250µM	250µM	25µM

Table 5. Deaza dGTP Nucleotide Mix Formulations for *Taq* DNA Polymerase.

Component	G Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	C Nucleotide Mix
ddGTP	25µM	—	—	—
ddATP	—	350µM	—	—
ddTTP	—	—	300µM	—
ddCTP	—	—	—	160µM
7-deaza dGTP	25µM	250µM	250µM	250µM
dATP	250µM	25µM	250µM	250µM
dTTP	250µM	250µM	25µM	250µM
dCTP	250µM	250µM	250µM	25µM

III. *Taq* DNA Polymerase Sequencing with the Two-Step Extension/Labeling Protocol

Reagents to be Supplied by the User

- [α-^{35}S]dATP (1,000Ci/mmol) or [α-^{32}P]dATP (800Ci/mmol)

A. Annealing The Template and Primer

1. Annealing Single-Stranded DNA Template and Primer

Single-stranded DNA can be prepared from M13 or phagemid vectors (Section IX, pg. 109). Anneal the primer with ssDNA template in an approximately 1:1 molar ratio. For each set of four sequencing reations, mix the following reagents in a microcentrifuge tube:

ssDNA (approx. 2µg of an M13 template)	0.8pmol
primer (approx. 8ng of a 24mer; Table 2, pg. 80)	1.0pmol
Taq DNA polymerase 5X buffer (pg. 81)	5.0µl
extension/labeling mix	2.0µl
sterile H_2O	to final volume 25µl

Incubate at 37°C for 10 minutes. While this reaction is incubating, prepare the nucleotide tubes for the termination reaction as described in Step C.1, below.

2. Annealing Double-Stranded Plasmid Template and Primer

Prior to annealing, alkali denature and precipate the template (Section VIII.C, pg. 107). Anneal the primer with the dsDNA plasmid template in an approximately 1:1 molar ratio. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

denatured plasmid dsDNA (approx. 4mg of a 3-5kb template)	1.6pmol
primer (approx. 16ng of a 24mer: Table 2, pg. 80)	2pmol
Taq DNA polymerase 5X buffer (pg. 81)	5.0µl
extension/labeling mix	2.0µl
sterile H_2O	to final volume 25µl

Incubate at 37°C for 10 minutes. While the annealing reaction is incubating, prepare the nucleotide tubes for the termination reaction as described in Step C.1, below.

3. Annealing Lambda Template and Primer

The recommended amount of double-stranded lambda template to use per set of sequencing reactions is 0.4pmol, which represents 10µg of an approximately 42kb lambda vector. Prior to annealing, alkali denature and precipitate the template (Section VIII.D, pg. 108). The radiolabeled primer is annealed with the dsDNA lambda template in an approximately 5:1 molar ratio. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

denatured lambda dsDNA (approx. 10µg of a 42kb template)	0.4pmol
Taq DNA polymerase 5X buffer (pg. 81)	5.0µl
primer (2pmol)	1.0µl
sterile H_2O	to final volume 23µl

Heat to 95°C for 5 minutes to denature any secondary structure, then allow to anneal at 37°C for 10 minutes. Add 2µl of extension/labeling mix. While the annealing reaction is incubating, prepare the termination reaction tubes as described on pg. 84, Steps 1 and 2.

B. Extension/Labeling Reaction

1. Add 2µl of [α-^{35}S]dATP (1,000Ci/mmol, approximately 10µCi/µl) or 1µl of [α-^{32}P]dATP (800Ci/mmol, approximately 10µCi/µl) to the annealed primer/template mixture.
2. Add 1.6µl of sequencing grade *Taq* DNA polymerase (2.5u/µl) and mix briefly by pipetting up and down.
3. Incubate at 37°C for 5 minutes.

Note: The extension/labeling reaction is carried out at 37°C rather than 70°C to slow down the incorporation rate of *Taq* DNA polymerase and thereby limit the number of bases incorporated in this step. This allows sequence to be read close to the primer. To read closer to the primer, shorten the duration of the extension/labeling reaction.

Nucleic Acid Sequencing and Mutagenesis

III. *Taq* DNA Polymerase Sequencing with the Two-Step Extension/Labeling Protocol

(continued)

C. Termination Reaction

1. For each set of sequencing reactions, label four microcentrifuge tubes (G, A, T, C) and add 1µl of the appropriate d/ddNTP mix to each tube. Store on ice or at 4°C until just before completion of the extension/labeling reaction.

2. When the extension/labeling reaction is complete, aliquot 6µl to each tube (G,A,T,C) containing d/ddNTP mix. Mix briefly by pipetting up and down. A brief spin may be needed to ensure that no liquid is left on the tube walls.

3. Incubate at 70°C for 15 minutes.

4. Add 4µl of stop solution to each tube and set at room temperature.

5. Heat the reactions to ≥70°C for 2-5 minutes immediately before loading on a sequencing gel. Load 2.5-3.0µl of each reaction on the gel.

Notes:

1. Reactions can be stored overnight at –20°C or –70°C. [α-^{35}S]dATP labeled reactions can be stored at –70°C for 2 weeks.

2. The calculated Tm for a sequencing primer is often less than 70°C, the temperature of the sequencing reaction. However, this discrepancy does not result in interference with primer annealing and extension. A likely explanation for this observation is that primer/template complexes annealed at 37°C are rapidly stabilized by extension with *Taq* DNA polymerase as the reaction warms to 70°C.

3. When sequencing lambda templates, sequence data closer to the primer can be obtained with the one-step end-labeled primer procedure.

4. To detect lambda sequencing products from the two-step extension/labeling protocol, expose ^{32}P-labeled products to film overnight with an intensifying screen and ^{35}S-labeled products for 2-3 days without a screen.

IV. Klenow and Reverse Transcriptase Sequencing Methods

Reagents to be Supplied by the User

- [α-^{32}P]dATP or [α-^{35}S]dATP (400-500Ci/mmol)

A. Radioisotope Considerations

The Klenow and reverse transcriptase systems are optimized for use with either [α-^{32}P]dATP or [α-^{35}S]dATP at specific activities in the range of 400-500Ci/mmole. Higher specific activities can be used with equivalent results if the reactions are supplemented with unlabeled dATP. The optimal concentration of the unlabeled dATP needed to compensate for the higher specific activity label will vary depending on both the source and the specific activity of the label. If higher specific activity label is used, we recommend that a preliminary experiment be performed in which the A reaction is supplemented with a final concentration of unlabeled dATP from 0–20µM and run on a sequencing gel. Inadequate concentrations of unlabeled dATP will result in early termination of the A reaction while excess amounts will only generate sequence in the upper portion of the gel. We have found that a final concentration of 4-10µM unlabeled dATP appears to be optimal for adjustments of the deoxy/dideoxynucleotide ratios with >3,000Ci/mmole dATP label.

Since the nucleotide mixes may be used with either [α-^{32}P]dATP or [α-^{35}S]dATP, it is necessary to determine which label is appropriate for the researcher's particular needs. Each radioisotope has unique advantages. Using [α-^{32}P]dATP in the sequencing reaction ensures 120 bases of readable sequence with a single load and an overnight exposure of the autoradiogram. Improved base resolution and extended read length, upward of 150 bases with a single load, are the advantages of using [α-^{35}S]dATP. (Using multiple gel loadings, sequence data can be read to >350 bases with these nucleotide mixes.) A significant consideration when using [α-^{35}S]dATP is that exposure times up to 60-80 hours may be required for an adequately exposed autoradiograph.

IV. Klenow and Reverse Transcriptase Sequencing Methods

(continued)

B. Annealing the Primer with Single-Stranded DNA or RNA Templates

1. In a siliconized screw cap microcentrifuge tube, place 1-2µg of single-stranded DNA or RNA template, 1µl of the appropriate 10X buffer (Klenow or RT), 3µl of the appropriate primer (stock 10ng/µl), and water to make the final volume 10µl. **Note:** Use RT for sequencing RNA. Either Klenow or RT may be used for sequencing DNA.
2. Heat the annealing reactions for 3 minutes at 85°C. Allow to cool at room temperature. For sequencing single-stranded RNA, anneal the template and primer for 3 minutes at 67°C and allow to cool at room temperature.
3. Centrifuge for 2 seconds in a microcentrifuge to recover the contents. Proceed to Section D or E, below.

C. Annealing the Primer with Double-Stranded Plasmid DNA

The recommended amount of double-stranded plasmid template to use per set of sequencing reactions is approximately 1pmol or about 2µg of a 3-5kb plasmid. Prior to annealing, alkali denature and precipitate the template (Section VIII.C, pg. 107).

1. For each set of four sequencing reactions, mix the following reagents in a microcentrifuge tube:

denatured plasmid dsDNA (approx. 2-3µg of a 3-5kb template)	0.5-1pmol
Klenow or RT enzyme 10X buffer (pg. 86)	1µl
primer (Table 2, pg. 80)	1.6pmol
H_2O	to final volume 10µl

2. Mix by pipetting the solution up and down a few times and incubate at 37°C for 15 minutes.

D. Klenow Sequencing Method

1. Prepare four screw cap siliconized tubes labeled C, A, T, and G for each set of sequencing reactions. Add 3µl of the appropriate Klenow nucleotide mix to each tube.
2. Add 5 units of Klenow to each annealing mixture. Mix by pipetting up and down a few times.
3. Add 4µl of [α-^{32}P]dATP or 5µl of [α-^{35}S]dATP to the annealing reaction. Pipet up and down a few times to mix.
4. Add 3µl of the label/primer/template mix to each of the nucleotide tubes and incubate at 37°C for 15 minutes for [α-^{32}P]dATP labeled reactions or 20 minutes for [α-^{35}S]dATP labeled reactions.
5. Add 1µl of chase solution to each tube. Incubate at 37°C for another 15 minutes.
6. Stop the reactions by adding 5µl of stop solution.
7. Heat the reactions at 70°C for 3 minutes before loading the sequencing gel. Load 2.5µl of each reaction onto the sequencing gel.

Note: Reactions can be stored at –20°C or –70°C overnight. [α-^{35}S]dATP labeled reactions can be stored at –70°C for 2 weeks.

Nucleic Acid Sequencing and Mutagenesis

IV. Klenow and Reverse Transcriptase Sequencing Methods

(continued)

E. Reverse Transcriptase Sequencing Method

1. Prepare four microcentrifuge tubes labeled C, A, T, and G for each set of sequencing reactions. Add 3µl of the appropriate RT nucleotide mix to each tube.

2. Add 5 units of RT to each annealing mixture. Mix by pipetting up and down a few times.

3. Add 4µl of [α-^{32}P]dATP or 5µl of [α-^{35}S]dATP to the annealing reaction. Pipet up and down a few times to mix.

4. Add 3µl of the label/primer/template mix to each of the nucleotide tubes and incubate at 42°C for 15 minutes for [α-^{32}P]dATP labeled reactions or 20 minutes for [α-^{35}S]dATP labeled reactions.

5. Add 1µl of chase solution to each tube. Incubate at 42°C for another 15 minutes.

6. Stop the reactions by adding 5µl of stop solution.

7. Heat the reactions to ≥70°C for 2-5 minutes before loading on the sequencing gel. Load 2.5µl of each reaction on the sequencing gel.

Note: Reactions can be stored at –20°C or –70°C overnight. [α-^{35}S]dATP labeled reactions can be stored at –70°C for 2 weeks.

Composition of Solutions

Klenow 10X buffer (supplied):

100mM	Tris-HCl, pH 7.5
500mM	NaCl

RT 10X Buffer (supplied):

340mM	Tris-HCl, pH 8.3
500mM	NaCl
50mM	$MgCl_2$
50mM	DTT

Chase solution (supplied):

34mM	Tris-HCl, pH 8.3
50mM	NaCl
6mM	$MgCl_2$
5mM	DTT
2mM	dATP
2mM	dCTP
2mM	dTTP
2mM	dGTP

Stop solution (supplied):

98%	formamide
10mM	EDTA
0.1%	xylene cyanol
0.1%	bromophenol blue

IV. Klenow and Reverse Transcriptase Sequencing Methods

(continued)

Table 6. Klenow Nucleotide Mix Formulations.*

Component	C Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	G Nucleotide Mix
ddCTP	16.7μM	—	—	—
ddATP	—	100μM	—	—
ddTTP	—	—	117μM	—
ddGTP	—	—	—	16.7μM
dATP	—	—	—	—
dCTP	1.66μM	33μM	33μM	33μM
dTTP	33μM	33μM	1.66μM	33μM
dGTP	33μM	33μM	33μM	1.66μM

*The Klenow C, A, T, and G nucleotide mixes also contain 10mM Tris-HCl, pH 7.5, 50mM NaCl, 10mM $MgCl_2$, and 1mM DTT. Klenow chase solution contains 34mM Tris-HCl, pH 8.3, 50mM NaCl, 6mM $MgCl_2$, and 5mM DTT.

Table 7. Reverse Transcriptase Nucleotide Mix Formulations.*

Component	C Nucleotide Mix	A Nucleotide Mix	T Nucleotide Mix	G Nucleotide Mix
ddCTP	12.5μM	—	—	—
ddATP	—	1.0μM	—	—
ddTTP	—	—	50μM	—
ddGTP	—	—	—	12.5μM
dATP	—	—	—	—
dCTP	250μM	250μM	250μM	250μM
dTTP	250μM	250μM	250μM	250μM
dGTP	250μM	250μM	250μM	250μM

*The reverse transcriptase C, A, T, and G nucleotide mixes and chase solution also contain 34mM Tris-HCl, pH 8.3, 50mM NaCl, 6mM $MgCl_2$, and 5mM DTT.

Nucleic Acid Sequencing and Mutagenesis

V. Sequencing of RNA Transcripts with the GemSeq® Transcript System

The GemSeq® transcript sequencing system may be used to sequence a wide variety of RNA molecules, and is especially tailored for sequencing RNA transcripts generated *in vitro* from Promega's pGEM® and pGEMEX™ vectors. Following transcription, the appropriate promoter primer is annealed to the RNA and serves as the priming site for AMV reverse transcriptase. Primed reverse transcripts are truncated during the reaction using dideoxy nucleotide analogues. With the GemSeq transcript system, the first base following the promoter can be easily observed in standard gel systems. This permits verification of the integrity of the insert ends using restriction sites as marker sequences. Other RNA sequencing applications include viral RNAs and ribosomal RNAs (11). The system can be used with either [α-^{32}P]dATP or [α-^{35}S]dATP as the label.

Materials to be Supplied by the User

- TE-saturated phenol/chloroform (pg. 89)
- TE buffer (pg. 89)
- 3M sodium acetate
- ethanol (100% and 70%)
- [α-^{32}P]dATP or [α-^{35}S]dATP (400Ci/mmol)

A. Preparation of RNA Transcripts

1. Gloves should be worn when working with the transcription reagents or the transcript to prevent RNase contamination. The transcription reaction can be done in different volumes depending on the amount of transcripts desired. A 20µl reaction will yield enough template for four sets of sequencing reactions (approximately 4µg of transcript) and a 50µl reaction will yield enough template for 10 sets of sequencing reactions (approximately 10µg of transcript). In a sterile 1.5ml microcentrifuge tube, combine the reagents as follows:

	20µl reaction	50µl reaction
transcription 5X buffer* (pg. 89)	4.0µl	10.0µl
2.5mM each of ATP, CTP, UTP, and GTP (made by mixing together equal amounts of the 10mM rNTP stocks supplied*)	4.0µl	10.0µl
100mM DTT*	2.0µl	5.0µl
pGEM® or pGEMEX™ recombinant plasmid (1µg/µl stock)	1.0µl	2.0µl
SP6, T7 or T3 RNA polymerase	5-10u	10-20u
DEPC-treated H_2O	to final volume 20µl	50µl

*These reagents are available as pre-tested components in the Riboprobe® Gemini system II buffer kit.

2. Incubate at 37°C for 30 minutes. Meanwhile, set up the annealing reactions as described in Section B, pg. 89.

3. If the transcripts are used immediately, nothing needs to be done to stop the transcription reaction after the 30 minute incubation. Discard any of the transcript reaction remaining at the end of the day. If storage of the transcripts is desired, the transcription reaction can be extracted with one volume of TE-saturated phenol/chloroform (pg. 89). Vortex for 1 full minute and centrifuge at 12,000 x g for 5 minutes.

4. Transfer the upper, aqueous phase to a fresh tube. Add an equal volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 full minute and centrifuge at 12,000 x g for 5 minutes.

5. Transfer the upper, aqueous phase to a fresh tube and add 1/10 volume of 3M sodium acetate and 2 volumes of ethanol. Mix and leave at –20°C or on dry ice for 30 minutes.

6. Centrifuge at 12,000 x g for 15 minutes. Remove the supernatant and carefully rinse the pellet with 70% ethanol. Drain the tube and dry the pellet under vacuum.

7. Resuspend in deionized H_2O. Store at –20°C or –70°C.

   Note: The transcript size can be checked by electrophoresis of 5µl of the transcript on a 3% PAGE/7M urea gel. Using a circular template, the transcript should be heterogeneous and much larger than the insert to be sequenced.

Promega

V. Sequencing of RNA Transcripts with the GemSeq® Transcript System

(continued)

B. Annealing the Primer with Template RNA

We recommend using screw cap polypropylene microcentrifuge tubes for the annealing and sequencing reactions. The screw caps prevent possible water leakage into the annealing reaction and contain the radioactivity more effectively when the tubes are spun in a microcentrifuge.

1. In a screw cap tube, mix:

deionized H_2O	1µl
RT 10X buffer	1µl
SP6 or T7 promoter primer* (10ng/µl)	3µl
RNA transcript from Step A, pg. 88 (1µg)	5µ
final volume	10µl

(The first three components may be combined in advance and left on ice until the transcription reaction is complete).

*If SP6 RNA polymerase was used in the transcription, the T7 promoter primer is used for sequencing. Conversely, if T7 RNA polymerase was used in the transcription, the SP6 promoter primer is used for sequencing.

2. Heat the annealing reactions to 65°C and then place at room temperature to cool. While the annealing reactions are cooling, prepare the tubes for the sequencing reactions (Section C, below).

3. Centrifuge for 2 seconds in a microcentrifuge to recover the contents.

C. Transcript Sequencing Reactions

1. While the annealing reactions are cooling, prepare tubes for each reaction and label them C, A, T and G.

2. Add 2.5µl of the RT nucleotide mix to each tube and store at 0-4°C.

3. When the temperature of the annealing reaction is about 48°C, thaw the [α-^{32}P]dATP (>400Ci/mmole, 10µCi/µl) or [α-^{35}S]dATP (>400Ci/mmole, 10µCi/µl).

4. After centrifugation of the annealing reaction tubes (Section B, step 4), add 5 units of AMV reverse transcriptase to each annealing mixture. Mix by pipetting up and down a few times.

5. Add 2.5µl of [α-^{32}P]dATP or 4µl of [α-^{35}S]dATP to each annealing mixture. Mix by pipetting up and down a few times.

6. Add 3.0µl of the primer/template/label/reverse transcriptase mixture to each of the nucleotide mixtures and incubate at 42°C for 15 minutes (^{32}P-labeled reactions) or 20 minutes (^{35}S-labeled reactions).

7. Add 1µl of chase solution to each reaction. Incubate at 42°C for an additional 15 minutes.

8. Stop the reactions by the addition of 5µl of stop solution.

9. Heat the reaction mixtures at 70°C for 5 minutes before loading on the sequencing gel. Load 2.5µl of each reaction on the sequencing gel.

Note: Reactions can be stored overnight at –20°C or –70°C. [α-^{35}S]dATP labeled reactions can be stored at –70°C for 2 weeks.

Solutions Required

Transcription 5X buffer:

200mM	Tris-HCl, pH 7.5
30mM	$MgCl_2$
10mM	spermidine
50mM	NaCl

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

RT 10X buffer:

340mM	Tris-HCl, pH 8.3
500mM	NaCl
50mM	$MgCl_2$
50mM	DTT

TE-saturated phenol/chloroform

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

*Buffer tablets available from Promega.

Nucleic Acid Sequencing and Mutagenesis

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

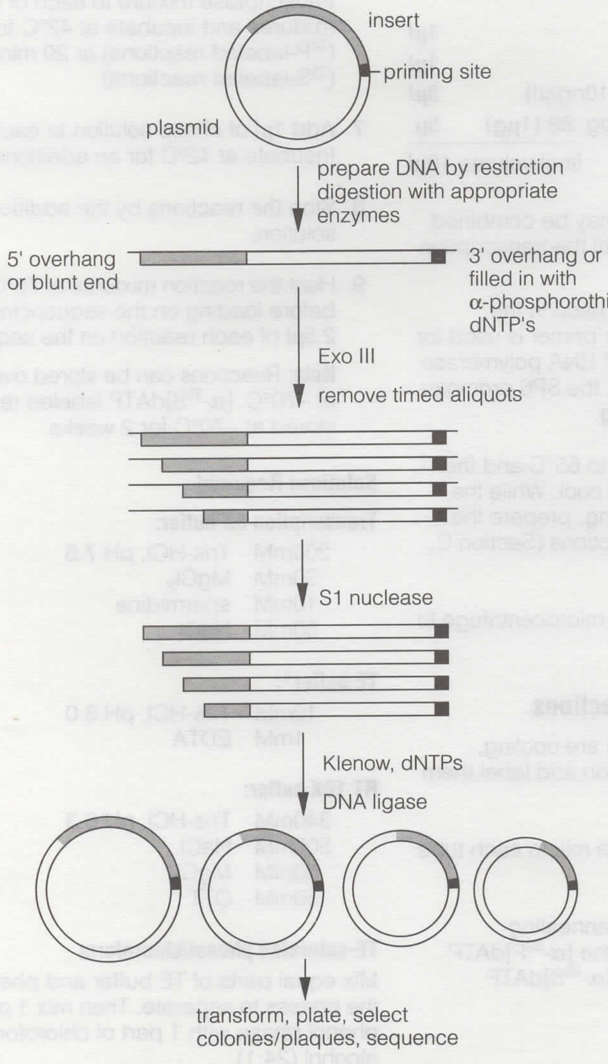

Figure 1. Schematic diagram of the Erase-a-Base system.

Technical Services: **800-356-9526**

A. General Considerations

The Erase-a-Base® system is designed for the rapid construction of plasmid or M13 subclones containing progressive unidirectional deletions of any inserted DNA, thus allowing efficient sequence analysis of large DNA fragments. The system is based on the procedure developed by Henikoff (12), in which exonuclease III (Exo III) is used to digest DNA from a 5′ protruding or blunt end, while leaving a 4 base 3′ protruding end or an α-phosphorothioate filled end (13) intact. The uniform rate of digestion of the enzyme allows a series of deletions of increasing size to be made by removing timed aliquots from the reaction. Starting with appropriately treated plasmid or M13 DNA, a collection of unidirectional deletions spanning several kb can be easily constructed in several hours. All treatments are performed by successive additions of the various reagents, thus avoiding cumbersome extraction or precipitation of multiple samples.

A schematic diagram of the steps involved in the method is shown in Figure 1. The DNA fragment of interest is first cloned into the multiple cloning site of an appropriate vector (e.g., a pGEM® plasmid), so that at least two unique restriction sites lie between the end of the insert to be deleted and the sequencing primer binding site (either the SP6 or T7 promoter, in the case of the pGEM plasmids). The enzyme that cuts closest to (or within) the insert DNA must leave blunt ends or 5′ overhangs, susceptible to Exo III. When this configuration of sites is not convenient, then an enzyme producing a 5′ overhang can be used closest to the priming site, followed by filling in the recessed 3′ ends with alpha-phosphorothioate deoxynucleotides. This treated end is also resistant to Exo III digestion (2).

Exo III digestion of the doubly cut DNA proceeds synchronously from the blunt or 5′ unprotected end into the insert. Samples are removed at timed intervals to tubes containing S1 nuclease, which removes the single-stranded tails remaining after Exo III digestion. The low pH and the presence of zinc cations in the S1 buffer effectively inhibit further digestion by Exo III. S1 nuclease, unlike mung bean nuclease, is active in the Exo III buffer conditions, so it is not necessary to change buffers between the Exo III and S1 steps. After neutralization and heat inactivation of the S1 nuclease, Klenow DNA polymerase is added to generate blunt ends, which are then ligated to circularize the deletion-containing plasmids. The ligation mixtures are used directly to transform competent cells. Each successive time

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

point yields a collection of subclones containing clustered deletions extending further into the original insert.

A number of subclones from each time point are then screened to select for appropriate intervals between deletions. Sequence analysis can be conveniently performed directly with double-stranded plasmid constructs using the TaqTrack® sequencing system with SP6 or T7 promoter primers (for pGEM vector plasmids), or other appropriate sequencing primers.

The Erase-a-Base system can be used with any plasmid or M13 vector. The pGEM-5Zf and pGEM-7Zf vectors, however, have been designed specifically for use with the Erase-a-Base system. The multiple cloning region in these vectors is flanked by 3′ overhang restriction enzyme sites which protect the SP6 and T7 RNA polymerase promoters from digestion with exonuclease III. For circle maps of these vectors, see the Vector Maps chapter. Further information on the Erase-a-Base system can be found in the Erase-a-Base System Technical Manual and in reference 12.

B. Preparation of Closed Circular DNA

The generation of ordered sets of deletions by this method relies on the uniform digestion rate of exonuclease III from appropriate DNA ends. However, this enzyme also digests from nicks in double-stranded DNA molecules (14), creating single-stranded gaps. The effect of random nicks in the starting DNA is to randomize the resulting deletions obtained. The greater the percentage of nicked molecules in the starting material, the more random the deletions become and the more difficult it becomes to screen for the desired (predicted) deletions among the resulting subclones. Therefore, it is important to minimize the proportion of nicked molecules in the starting DNA. This can be accomplished by minimizing the amount of nicked (and linear) molecules in the plasmid preparation and minimizing the generation of single-stranded nicks during restriction enzyme digestions.

Closed circular DNA can be purified by conventional equilibrium sedimentation in CsCl-ethidium bromide gradients (15). Alternatively, nicked and linear DNA can be selectively removed from supercoiled DNA by acid-phenol extraction as described by Zasloff, *et al.* (16). This procedure is presented below.

Reagents to be Supplied by the User

- 50mM sodium acetate, pH 4.0
- 3M sodium acetate, pH 5.2
- 2M sodium acetate, pH 4.0
- 1M Tris-HCl, pH 8.6
- 2M NaCl
- redistilled phenol
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- TE buffer (pg. 97)

1. Preparation of Acid-Phenol

a. Add 500ml of 50mM sodium acetate, pH 4.0, to 500g of melted phenol and stir to dissolve (approximately 3-4 hours at room temperature).

b. Let the phases separate and remove the upper, aqueous phase by aspiration. Add 500ml of 50mM sodium acetate, pH 4.0, and stir to emulsify. Let the phases separate again.

c. Remove a small sample of the aqueous phase and determine the pH using a pH meter.

d. Repeat Step 1.b 2-3 times or until the pH of aqueous buffer is less than 4.1 (see Note 2). Store at 4°C.

2. Acid-Phenol Extraction

a. Carry out the preparation of plasmid or replicating form DNA by standard methods so that the DNA is free of RNA and protein (15).

b. Add 0.1 volume of 3M sodium acetate, pH 5.2, and precipitate by adding 2 volumes of ethanol. Rinse the pellet with 70% ethanol and dry under vacuum.

c. Dissolve the pellet in deionized water and then add 2M sodium acetate, pH 4.0, to give a final concentration of 50mM and 2M NaCl to give a final concentration of 75mM.

d. Add an equal volume of acid-phenol (prepared above) and mix thoroughly. Centrifuge at 10,000 x g for 5 minutes and recover the aqueous phase. The appearance of the lower phase can vary from a turbid white emulsion to having a thick white band near the interface. Two or three extractions may be needed to remove most of the contaminating nicked and linear DNA.

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

e. When the extraction is complete, add 0.05 volume of 1M Tris-HCl, pH 8.6, and extract with 1 volume of chloroform:isoamyl alcohol (24:1).

f. Recover the aqueous phase, add 0.1 volume of 2M NaCl, and precipitate with ethanol. Rinse the pellet with 70% ethanol, dry under vacuum, and dissolve the DNA at 0.5-1.0µg/ml in TE buffer for storage.

Notes:

1. The mechanism by which this procedure selectively removes nicked and linear DNA is not as yet understood. However, the method is very reproducible and should allow good recovery of closed circular DNA.

2. It is very important that the pH of the phenol be 4.0; no selective removal is seen at pH 4.2. In addition, low ionic strength must be maintained because the closed circular DNA partitions into the phenol phase as the NaCl concentration is raised above 125mM.

3. The time that the DNA is exposed to low pH should be minimized since this can result in depurination, causing losses in the recovery of closed circular molecules. The extraction procedure will work either at 4°C or room temperature, but Zasloff, *et al.* (16) recommend 4°C to reduce the rate of depurination. Their results indicate that, using the recommended conditions, nicking occurred at a rate of less than one purine per 30kbp per hour.

C. Restriction Digestion of Plasmid DNA

When possible, double cut 5-10µg of closed circular DNA with two different restriction enzymes: one that generates a 4-base 3′ protrusion protecting the primer binding site and another which leaves a 5′ protrusion or blunt end adjacent to the insert from which deletions are to proceed (see Tables 8 and 9, pg. 93). **Note that *Apa* I, *Pst* I and *Sac* II generate 3′ protruding ends, but these are not resistant to Exo III digestion.** If salt conditions do not allow a double digestion, digest with the restriction enzyme that cuts at the lower salt concentration, then add the second restriction enzyme and additional salt. A table of NaCl concentrations for multiple restriction digestions is provided on pg. 23. Since Exo III is strongly inhibited by as little as 20mM NaCl (17), the plasmid DNA must be extracted with phenol/chloroform and precipitated with ethanol following restriction digestion.

The original vector preparation should not contain any nicked molecules. See pg. 91, Section B, for ways to minimize the proportion of nicked molecules in the starting DNA.

Reagents to be Supplied by the User

- 2M NaCl
- restriction enzymes and appropriate buffers
- TE-saturated phenol/chloroform (pg. 97)
- ethanol (100% and 70%)
- 0.1M DTT

Protocol

1. The following restriction digestion reaction is provided as an example:

recombinant plasmid DNA	5µg
appropriate restriction enzyme 10X buffer	5µl
1mg/ml acetylated BSA (optional)	5µl
appropriate restriction enzyme	5-25u
deionized H_2O	to final volume 50µl

Incubate at the appropriate temperature for 2-3 hours.

2. Check that the reaction has gone to completion by electrophoresis of a sample (0.3µg) on a 1% agarose minigel.

3. If the digest is complete, extract with 1 volume of TE-saturated phenol/chloroform (pg. 97). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 3.

5. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 2M NaCl and 2 volumes of ethanol. Mix and place at –20°C or on dry ice for 10 minutes. Centrifuge at 12,000 x g for 5 minutes.

6. Carefully pour off the supernatant and wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and proceed to pg. 94, Section E.

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

Table 8. Restriction Enzymes That Generate Exonuclease III Resistant 3′ Overhangs.

Enzyme	Recognition Sequence
***Aat* II**	5′ . . . GACGTC . . . 3′ 3′ . . . CTGCAG . . . 5′
Ban II	5′ . . . GPuGCPyC . . . 3′ 3′ . . . CPyCGPuG . . . 5′
Bgl I	5′ . . . GCCNNNNNGGC . . . 3′ 3′ . . . CGGNNNNNCCG . . . 5′
***BstX* I**	5′ . . . CCANNNNNNTGG . . . 3′ 3′ . . . GGTNNNNNNACC . . . 5′
Hae II	5′ . . . PuGCGCPy . . . 3′ 3′ . . . PyCGCGPu . . . 5′
Hha I	5′ . . . GCGC . . . 3′ 3′ . . . CGCG . . . 5′
***Kpn* I**	5′ . . . GGTACC . . . 3′ 3′ . . . CCATGG . . . 5′
***Nsi* I**	5′ . . . ATGCAT . . . 3′ 3′ . . . TACGTA . . . 5′
Pvu I	5′ . . . CGATCG . . . 3′ 3′ . . . GCTAGC . . . 5′
***Sac* I**	5′ . . . GAGCTC . . . 3′ 3′ . . . CTCGAG . . . 5′
***Sph* I**	5′ . . . GCATGC . . . 3′ 3′ . . . CGTACG . . . 5′

Notes:

1. The enzymes shown in bold cut in the multiple cloning regions of the pGEM-5Zf or pGEM-7Zf plasmids.
2. *Apa* I, *Pst* I and *Sac* II generate 3' overhangs, but these ends are not protected from exonuclease III digestion.

Table 9. Restriction Enzymes That Generate 5′ Overhangs or Blunt Ends in the pGEM-5Zf and pGEM-7Zf Plasmids.

Enzyme	Recognition Sequence
***Bam*H I**	5′ . . . GGATCC . . . 3′ 3′ . . . CCTAGG . . . 5′
***Cla* I**	5′ . . . ATCGAT . . . 3′ 3′ . . . TAGCTA . . . 5′
***Csp*45 I**	5′ . . . TTCGAA . . . 3′ 3′ . . . AAGCTT . . . 5′
***Eco*R I**	5′ . . . GAATTC . . . 3′ 3′ . . . CTTAAG . . . 5′
***Eco*R V**	5′ . . . GATATC . . . 3′ 3′ . . . CTATAG . . . 5′
***Hind* III**	5′ . . . AAGCTT . . . 3′ 3′ . . . TTCGAA . . . 5′
***Nco* I**	5′ . . . CCATGG . . . 3′ 3′ . . . GGTACC . . . 5′
***Nde* I**	5′ . . . CATATG . . . 3′ 3′ . . . GTATAC . . . 5′
***Not* I**	5′ . . . GCGGCCGC . . . 3′ 3′ . . . CGCCGGCG . . . 5′
***Sal* I**	5′ . . . GTCGAC . . . 3′ 3′ . . . CAGCTG . . . 5′
***Sma* I**	5′ . . . CCCGGG . . . 3′ 3′ . . . GGGCCC . . . 5′
***Spe* I**	5' . . . ACTAGT . . . 3' 3′ . . . TGATCA . . . 5′
***Xba* I**	5' . . . TCTAGA . . . 3' 3′ . . . AGATCT . . . 5′
***Xho* I**	5′ . . . CTCGAG . . . 3′ 3′ . . . GAGCTC . . . 5′

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

D. Protection of 5′ Protruding Ends with α-Phosphorothioate Nucleotides

An alternate strategy to using 3′ protrusions to block Exo III digestion of vector sequences is to fill in 3′ recessed ends with alpha-phosphorothioate deoxynucleotides and Klenow fragment. This approach allows enzymes leaving 5′ protrusions to be used as protecting sites for the generation of deletions with the Erase-a-Base system. This protecting restriction site must be cleaved and filled in with α-phosphorothioate deoxynucleotides before the second restriction digestion is performed.

Reagents to be Supplied by the User

- 2M NaCl
- TE-saturated phenol/chloroform (pg. 97)
- ethanol (100% and 70%)
- α-phosphorothioate nucleotides
- 0.1M DTT

Protocol

1. Digest 5-10μg of recombinant DNA to completion with the enzyme chosen to generate the primer protecting site.
2. If Klenow fragment is active in the restriction digestion buffer, the fill-in reaction can be performed directly (Steps 5-7). If Klenow is not active in this buffer, the DNA should be extracted first as described in Steps 3-4.
3. Add 1 volume of TE-saturated phenol/chloroform (pg. 97), vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.
4. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 2M NaCl and 2 volumes of ethanol. Mix and leave at –20°C or on dry ice for 10 minutes. Centrifuge at 12,000 x g for 5 minutes.
5. Remove the supernatant and carefully rinse the pellet in 70% ethanol. Drain the tube and dry the pellet under vacuum.
6. Resuspend the pellet in 50-100μl of Klenow 1X buffer. Add a mixture of all four α-phosphorothioate deoxyribonucleoside triphosphates to a final concentration of 40μM each.
7. Add dithiothreitol (DTT) to 1mM and Klenow fragment to 50u/ml. Incubate for 10 minutes at 37°C.
8. Heat the sample for 10 minutes at 70°C to inactivate the Klenow fragment. If the second restriction enzyme digests well in the same buffer, it can be added directly. Otherwise, extract the DNA as in Steps 3-5 and resuspend it in the appropriate digestion buffer. After performing the second restriction digestion, extract the DNA as in Steps 3-5 and proceed to Section E, below.

E. Exo III Deletion, Ligation, and Transformation Procedures

The following detailed procedure is based on using 5μg of doubly cut plasmid DNA containing a 6.5kb insert, where 24 time points are to be taken. The amount of each solution to be used is determined by the amount of starting DNA and desired number of time points. Reaction volumes can be scaled up or down in proportion to those described below.

The rate of Exo III digestion can be altered simply by changing the incubation temperature (18). Using the recommended amount of Exo III (300-500 units), the digestion rate exhibits the temperature dependence shown in Figure 2. Successive deletions differing in size by 300-450bp are convenient for use with sequencing systems capable of reading 350-450bp, since these allow overlaps of 50-150bp.

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

Reagents to be Supplied by the User

Instructions for preparing these reagents are supplied on pg. 97.

- S1 nuclease mix
- Klenow mix
- ligase mix
- SOC medium
- LB plates
- ampicillin

Protocol

1. Dissolve the DNA in 60µl of Exo III 1X buffer. Meanwhile, add 7.5µl of S1 nuclease mix (pg. 97) to each of 24 small tubes (or use a conical bottom 96 well plate) and leave on ice.

2. Warm the DNA tube to the digestion temperature in a water bath. Digestion proceeds at about 450 bases/minute at 37°C. There is a 20-30 second lag for the reaction to begin. Add 300-500 units of Exo III, mixing as rapidly as possible. Remove 2.5µl samples at 30 second intervals into the S1 tubes on ice, pipetting up and down briefly to mix.

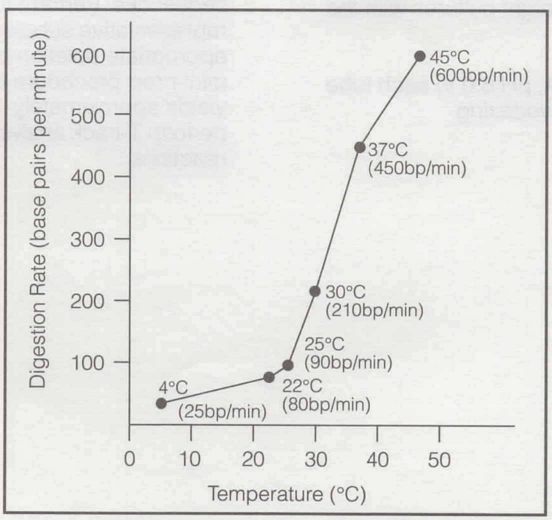

Figure 2. Temperature dependence of exonuclease III digestion rate.

3. After all the samples have been taken, move the tubes to room temperature for 30 minutes. Add 1µl of S1 stop buffer (pg. 97) and heat at 70°C for 10 minutes to inactivate the S1 nuclease.

4. To determine the extent of digestion, remove 2µl samples (about 40ng DNA) from each time point for analysis on a 1% agarose gel. By the time the samples are ready to be plated, the gel results will be available.

5. Transfer the samples from each time point to 37°C and add 1µl of Klenow mix (pg. 98) to each sample. Incubate for 3 minutes and then add 1µl of the dNTP mix (pg. 98). Incubate for 5 minutes more at 37°C.

6. Move the samples to room temperature and add 40µl of ligase mix (pg. 98) to each sample. Mix well and incubate at room temperature for 1 hour.

7. Thaw on ice 2-3 aliquots (200µl each) of competent cells of JM109 or another suitable strain. For each time point, add 10µl of ligation products to 200µl of competent cells, mix gently, and incubate for 30 minutes on ice (see Note 1) (8). Heat to 42°C for 45 seconds, then place on ice for 2 minutes. Add 200µl of SOC medium (pg. 98), warm to 37°C for 5 minutes, and shake at 37°C for 1 hour (9). Plate out the entire mixture on appropriate selective media. Promega's pGEM and other plasmids may be plated on LB plates containing 50µg/ml ampicillin (pg. 98). Using competent cells giving 10^7-10^8 transformants/µg supercoiled DNA, this procedure should result in dozens to hundreds of colonies per deletion time point.

Notes:

1. Competent cells are available from Promega. A procedure for preparing competent cells is also provided on pg. 52 of the Plasmid Cloning and Transcription *in vitro* chapter.

2. Other ligation conditions and transformation protocols can also be used. For example, PEG can be omitted from the ligase mix, but the incubations should then be carried out for several hours to overnight. If cells giving 10^6 or fewer transformants/µg are used, expect few colonies.

3. The use of SOC medium results in greater numbers of transformants than with LB medium.

Nucleic Acid Sequencing and Mutagenesis

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

F. Screening of Deletion Subclones

Screen a number of recombinants from each time point to select those containing deletions of appropriate sizes for further analysis. This can be accomplished by a number of rapid screening methods. One of these, a cracking procedure which allows rapid estimation of plasmid size, is described below (19).

After selecting the desired subclones, grow these up in overnight culture and perform a plasmid mini-prep (pg. 107, Section B) to obtain enough DNA for sequencing.

Reagents to be Supplied by the User

- cracking 2X buffer (pg. 98)
- 1% agarose gel with 1.5µg/ml ethidium bromide
- 10mM EDTA, pH 8.0

Rapid Screening Procedure

1. Using sterile toothpicks or pipet tips, pick individual colonies from plates, smear each colony near the bottom of a sterile microcentrifuge tube and streak the remaining cells on a fresh plate. Incubate the plate at 37°C. By the time the screening procedure is completed (2-3 hours), the streaks will have grown enough to allow inoculation of overnight cultures with the desired subclones.

2. Add 50µl of 10mM EDTA, pH 8.0 to each tube. Resuspend the cells by vortexing.

3. Add 50µl of freshly made cracking 2X buffer (pg. 98) to each tube and vortex. Incubate the tubes at 70° C for 5 minutes. Allow to cool to room temperature.

4. Add 1.5µl of 4M KCl and 0.5µl of 0.4% bromophenol blue and vortex.

5. Place on ice for 5 minutes. Spin in a microcentrifuge for 3 minutes at 4°C.

6. Run 25-50µl of the supernatant on a 0.7% agarose gel.

7. After the dye has migrated 3/4 the length of the gel, stain the gel by soaking it for 30-40 minutes in a solution of ethidium bromide (0.5µg/ml in water). Using appropriate supercoiled DNA markers, the plasmid sites can be estimated.

To determine the extent of sequence overlap in different deletion subclones, isolate plasmid DNA from each subclone of the desired size and screen by T-track analysis (20). In this technique, a sequencing reaction is performed on each subclone using only the dideoxy TTP reaction. The resultant banding pattern contains enough information to allow identification of overlapping sequences. Perform full sequence analysis on representative subclones that contain the appropriate deletion breakpoints. The plasmid mini-prep procedure described on pg. 107 (6) yields approximately 1-4µg of DNA, enough to perform T-track analysis or one set of sequencing reactions.

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VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

G. Troubleshooting

Problem	Recommendations
No apparent deletions occur when analyzed by gel electrophoresis.	If the restriction enzyme leaving the Exo III sensitive end failed to cut completely, those singly cut molecules will remain the same size as the original starting DNA throughout the deletion time course. Check the activity of the appropriate restriction enzyme. Repeat the initial digestion with more enzyme for a greater time period.
Deletion series appears to be degraded when analyzed by gel electrophoresis.	The original vector preparation contains nicked molecules. See page 91 for removal of nicked plasmid DNA.
Gel electrophoresis indicates secondary bands are present at each time point.	If the protecting restriction enzyme failed to cut completely, the singly cut molecules would be digested at twice the rate of those that were doubly cut so a second, smaller, species would be present at each time point. Check the activity of the appropriate restriction enzyme. Repeat the initial digestion utilizing more enzyme for a greater time period.
A low number of transformants are obtained.	Check the transformation efficiency of the competent cells by transforming intact supercoiled plasmid DNA. Bacterial cells must surpass 1 x 10^7 colonies/µg of supercoiled DNA transformed in order to obtain successful results with this system. If good transformation efficiency is obtained with the supercoiled DNA control, digest a sample of plasmid DNA with a restriction enzyme that generates a blunt end. Ligate several hundred nanograms of digested vector to itself using the components provided with the system. Load a sample on a low percentage agarose gel next to a sample of unligated linear vector and observe if there is an apparent shift in mobility of the ligated sample.

Solutions Required

Acid-phenol:

(Refer to Section B.1 for preparation)

TE buffer*:

10mM Tris-HCl, pH 8.0
1mM EDTA

TE-saturated phenol/chloroform

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Exo III 10X buffer:

660mM Tris-HCl, pH 8.0
6.6mM $MgCl_2$

S1 7.4X buffer:

0.3M potassium acetate, pH 4.6
2.5M NaCl
10mM $ZnSO_4$
50% glycerol

S1 nuclease mix (for 25 time points):

172µl deionized water
27µl S1 7.4X buffer
60u S1 nuclease

Make fresh for each experiment.

S1 stop buffer:

0.3M Tris base
0.05M EDTA

Klenow buffer:

20mM Tris-HCl, pH 8.0
100mM $MgCl_2$

VI. Generation of Unidirectional Deletions with the Erase-a-Base® System

(continued)

Klenow mix:

30µl	Klenow buffer
3-5u	Klenow DNA polymerase

Make fresh for each experiment.

dNTP mix:

0.125mM each of dATP, dCTP, dGTP, and dTTP

Ligase 10X buffer:

500mM	Tris-HCl, pH 7.6
100mM	$MgCl_2$
10mM	ATP

Ligase mix:

790µl	deionized water
100µl	ligase 10X buffer
100µl	50% PEG
10µl	100mM DTT
5u	T4 DNA ligase

Cracking 2X buffer (per 50ml):

2ml	5M NaOH
2.5ml	10% SDS
10g	sucrose

Add deionized H_2O to 50ml final volume.

*Buffer tablets available from Promega.

SOC medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl
10mM	$NaCl_4$
10Mm	$MgCl_2$
10mM	glucose

Adjust to pH 7.0 with NaOH. Autoclave.

LB plates with ampicillin (per liter):

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.0 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding ampicillin (50µg/ml final conc.). Pour 30-35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for one month).

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

A. General Considerations

Site-directed mutagenesis is a valuable tool for the study of DNA function and protein structure and function. A number of different mutagenesis methods have been reported (21,22). As introduced by Hutchinson, *et al.* (23), site-directed *in vitro* mutagenesis is accomplished by hybridizing to single-stranded DNA (ssDNA) a synthetic oligonucleotide which is complementary to the single-stranded template except for a region of mismatch near the center. It is this region that contains the desired nucleotide change or changes. Following hybridization with the single-stranded target DNA, the oligonucleotide is extended with DNA polymerase to create a double-stranded structure. The nick is then sealed with DNA ligase and the duplex structure is transformed into an *E. coli* host. Theoretically, the yield of mutants using this procedure should be 50% (due to the semi-conservative mode of DNA replication). In practice, however, the mutant yield may be much lower, often only a few percent or less. This is assumed to be due to such factors as incomplete *in vitro* polymerization, primer displacement by the DNA polymerase used in the fill-in reaction, and *in vivo* host-directed mismatch repair mechanisms which favor repair of the unmethylated newly synthesized DNA strand (24).

The Altered Sites™ *in vitro* mutagenesis system (Figure 3) consists of a unique mutagenesis vector and a simple, straightforward procedure for selection of oligonucleotide-directed mutants. The system is based on the use of a second mutagenic oligonucleotide to confer antibiotic resistance to the mutant DNA strand. The system employs a phagemid, the pSELECT™-1 vector, (pg. 366) which contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is always functional. The other, for ampicillin resistance, has been inactivated. An oligonucleotide is provided which restores ampicillin resistance to the mutant strand during the

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800-356-9526

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

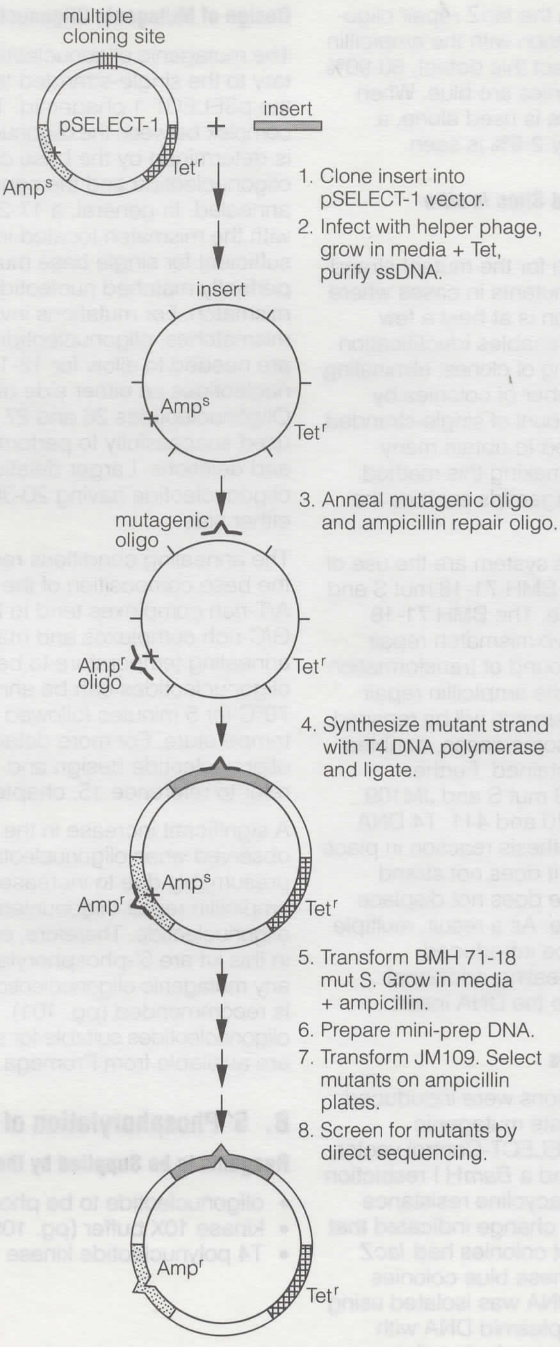

Figure 3. Schematic diagram of the Altered Sites *in vitro* mutagenesis procedure.

mutagenesis reaction. This oligonucleotide is annealed to the single-stranded DNA template at the same time as the mutagenic oligonucleotide and subsequent synthesis and ligation of the mutant strand links the two. The DNA is transformed into a repair minus strain of *E. coli* (BMH 71-18 mut S) and the cells are grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation in JM109 or a similar host ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants.

pSELECT™-1 Vector

The pSELECT-1 plasmid is a phagemid, defined as a chimeric plasmid containing the origin of a single-stranded DNA bacteriophage. This phagemid produces ssDNA upon infection of the host cells with the helper phage R408 or M13KO7 (25,26,27). The vector contains a multiple cloning site flanked by the SP6 and T7 RNA polymerase promoters and inserted into the *lac*Z α-peptide. Cloning of a DNA insert into the multiple cloning site results in inactivation of the α-peptide. When plated on indicator plates, colonies containing recombinant plasmids are white in a background of blue colonies. The SP6 and T7 promoters may be used to generate high specific activity RNA probes from either strand of the insert DNA. These sites also serve as convenient priming sites for sequencing of the insert. The pSELECT-1 vector carries gene sequences for both ampicillin and tetracycline resistance. However, the plasmid is ampicillin sensitive because a frameshift was introduced into this resistance gene by removing the *Pst* I site. Therefore, propagation of the plasmid and recombinants is performed under tetracycline selection. The sequence and a table of restriction sites for the pSELECT-1 vector are provided on pgs. 366-367.

pSELECT™-Control Vector

The pSELECT-Control vector provides a convenient white/blue positive control for mutagenesis reactions. This vector was derived from the pSELECT-1 vector by removing the *Pst* I site within the polylinker. The resultant frameshift in the *lac* α-peptide inactivated β-galactosidase and led to a white colony phenotype on indicator plates. A *lac*Z repair oligonucleotide (supplied with the system) may be used to introduce a four base insertion which corrects the defect in the *lac*Z gene and restores colony color to blue. The efficiency of the system in selecting for this repair mutation can be checked by the user; the fraction of blue colonies obtained gives the

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

mutagenesis efficiency. When the *lac*Z repair oligonucleotide is used in combination with the ampicillin repair oligonucleotide to correct this defect, 80-90% of the ampicillin resistant colonies are blue. When the *lac*Z repair oligonucleotide is used alone, a mutagenesis efficiency of only 2-5% is seen.

Important Features of the Altered Sites *in vitro* Mutagenesis System

The use of ampicillin selection for the mutant strand yields a high percentage of mutants in cases where the frequency without selection is at best a few percent. This high frequency enables identification of mutants by direct sequencing of clones, eliminating the need to screen large number of colonies by hybridization. Only a small amount of single-stranded DNA (0.1µg or less) is required to obtain many ampicillin resistant colonies, making this method ideally suited for use with phagemids such as the pSELECT-1 vector.

Two additional features of this system are the use of the repair minus *E. coli* strain BMH 71-18 mut S and the use of T4 DNA polymerase. The BMH 71-18 mut S strain suppresses *in vivo* mismatch repair (24,28). It is used in the first round of transformation to decrease the chance that the ampicillin repair mismatch or the mutagenic mismatch will be repaired. The use of this strain has, in some cases, doubled the percentage of mutants obtained. Further information on the BMH 71-18 mut S and JM109 strains is provided on pgs. 410 and 411. T4 DNA polymerase is used in the synthesis reaction in place of Klenow fragment because it does not strand displace (29,30) and therefore does not displace the mutagenic oligonucleotide. As a result, multiple site-directed mutations may be introduced simultaneously simply by annealing additional mutagenic oligonucleotides to the DNA insert.

Multiple Simultaneous Mutations

In one experiment, two mutations were introduced at the same time using separate mutagenic oligonucleotides. With the pSELECT-Control vector, *lac*Z function was restored and a *Bam*H I restriction site was generated in the tetracycline resistance gene. The white to blue color change indicated that 86% of the ampicillin resistant colonies had *lac*Z function restored. Fifteen of these blue colonies were selected and plasmid DNA was isolated using a mini-prep. Digestion of the plasmid DNA with *Bam*H I indicated that all fifteen colonies also contained the new *Bam*H I site.

Design of Mutagenic Oligonucleotides

The mutagenic oligonucleotide must be complementary to the single-stranded target DNA produced by the pSELECT-1 phagemid. The stability of the complex between the oligonucleotide and the template is determined by the base composition of the oligonucleotide and the conditions under which it is annealed. In general, a 17-20 base oligonucleotide with the mismatch located in the center will be sufficient for single base mutations. This gives 8-10 perfectly matched nucleotides on either side of the mismatch. For mutations involving two or more mismatches, oligonucleotides 25 bases or longer are needed to allow for 12-15 perfectly matched nucleotides on either side of the mismatch. Oligonucleotides 26 and 27 bases long have been used successfully to perform four base insertions and deletions. Larger deletions require an oligonucleotide having 20-30 matched bases on either side.

The annealing conditions required may vary with the base composition of the oligonucleotide. A/T-rich complexes tend to be less stable than G/C-rich complexes and may require a lower annealing temperature to be stabilized. Routinely, oligonucleotides can be annealed by heating to 70°C for 5 minutes followed by slow cooling to room temperature. For more detailed discussions of oligonucleotide design and annealing conditions, refer to reference 15, chapters 11 and 15.

A significant increase in the number of mutants is observed when oligonucleotides are phosphorylated, presumably due to increased linkage between the ampicillin repair oligonucleotide and the mutagenic oligonucleotide. Therefore, oligonucleotides included in this kit are 5′-phosphorylated. Phosphorylation of any mutagenic oligonucleotides used with this system is recommended (pg. 101). Custom phosphorylated oligonucleotides suitable for site-directed mutagenesis are available from Promega.

B. 5′ Phosphorylation of Oligonucleotides

Reagents to be Supplied by the User

- oligonucleotide to be phosphorylated
- kinase 10X buffer (pg. 105)
- T4 polynucleotide kinase

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

Protocol

1. Add the following components to a microcentrifuge tube.

oligonucleotide	100pmol
kinase 10X buffer	2.5µl
T4 polynucleotide kinase	5u
sterile H_2O	to final volume 25µl

2. Incubate the reaction at 37°C for 30 minutes.
3. Incubate the reaction at 70°C for 10 minutes to inactivate the kinase.
4. The reaction products can be stored at –20°C or added directly to the annealing reaction.

C. Cloning into the pSELECT-1 Vector and Preparation of pSELECT Single-Stranded DNA

Clone the DNA to be mutated into the pSELECT-1 vector using the multiple cloning sites shown on pg. 366. (Refer to pg. 51 for general information on cloning.) Transform the vector DNA into competent cells of JM109 or a similar host and select recombinant colonies by plating on LB plates containing 15µg/ml tetracycline, 0.5mM IPTG, and 40µg/ml X-Gal. After incubation for 24 hours at 37°C, colonies containing recombinant plasmids will appear white in a background of blue colonies. An alternative to preparing plates containing X-Gal and IPTG is to spread LB plates with 50µl of 50mg/ml X-Gal and 100µl of 100mM IPTG and allow these components to absorb for 30 minutes at 37°C prior to plating cells.

Note: The pSELECT-Control vector can also be grown on LB plates containing tetracycline and IPTG/X-Gal. Colonies will appear white, however, due to the defect in the *lac*Z gene.

To produce single-stranded template for the mutagenesis reaction, grow individual colonies containing pSELECT-Control or recombinant pSELECT-1 phagemids and infect the cultures with helper phage as described below. The single-stranded DNA produced is complementary to the strand of the multiple cloning site sequence shown on pg. 366.

Two helper phages, R408 and M13KO7, are offered as part of this system in order to provide the greatest latitude in optimizing ssDNA yields. Differences in the yields of ssDNA have been observed to be dependent on the particular combination of host, vector, and helper phage. In our experience, higher yields are obtained when the R408 helper phage is used in conjunction with the JM109 strain.

Reagents to be Supplied by the User

Instruments for preparing these solutions are provided on pg. 105.

- 7.5M ammonium acetate, pH 7.5
- TYP broth
- phage precipitation solution
- TE buffer
- TE-saturated phenol/chloroform
- ethanol (100% and 70%)

Protocol

1. Prepare an overnight culture of cells containing pSELECT-1 or pSELECT-Control phagemid DNA by picking individual tetracycline resistant colonies from a fresh plate. Inoculate 1-2ml of TYP broth (pg. 105) containing 15µg/ml tetracycline and shake at 37°C.
2. The next morning, inoculate 5ml of TYP broth containing 15µg/ml tetracycline with 100µl of the overnight culture. Shake vigorously at 37°C for 30 minutes in a 50ml flask.
3. Infect the culture with helper phage R408 or M13KO7 at an m.o.i. (multiplicity of infection) of 10 (i.e., add 10 helper phage particles per cell). For the helper phages supplied with this system, add 40µl. Continue shaking for 6 hours to overnight with vigorous agitation and good aeration.
4. Harvest the culture supernatant by pelleting the cells at 12,000 x g for 15 minutes. Pour the supernatant into a fresh tube and spin again for 15 minutes.
5. Precipitate the phage by adding 0.25 volume of phage precipitation solution (pg. 105) to the supernatant. Leave on ice for 30 minutes, then centrifuge for 15 minutes at 12,000 x g. Thoroughly drain the supernatant.

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VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

6. Resuspend the pellet in 400µl of TE buffer (pg. 105) and transfer the sample to a microcentrifuge tube.
7. Add 0.4ml of chloroform:isoamyl alcohol (24:1) to lyse the phage, vortex for 1 full minute, and centrifuge in a microcentrifuge (12,000 x g) for 5 minutes. This step removes excess PEG.
8. Transfer the upper, aqueous phase (containing phagemid DNA) to a fresh tube, leaving the interface behind. Add 0.4ml of TE-saturated phenol:chloroform (pg. 105) to the aqueous phase, vortex for 1 full minute, and centrifuge as in Step 7.
9. Transfer the upper, aqueous phase to a fresh tube and repeat the phenol extraction as in Step 8. If necessary, repeat this extraction several times until there is no visible material at the interface.
10. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume (200µl) of 7.5M ammonium acetate plus 2 volumes (1.2ml) of ethanol. Mix and leave at –20°C for 30 minutes to precipitate the phagemid DNA.
11. Centrifuge at 12,000 x g for 5 minutes, remove the supernatant, carefully rinse the pellet with 70% ethanol, and centrifuge again for 2 minutes. Drain the tube and dry the pellet under vacuum. The pellet may be difficult to see.
12. Resuspend the DNA in 20µl of H_2O. The amount of DNA present can be estimated by agarose gel electrophoresis of a 2µl sample.

    Two major bands are usually seen on 1% agarose gels run in Tris-acetate buffer: helper phage DNA and single-stranded plasmid DNA. In some preparations, a small amount of large chromosomal DNA may be present as well as some RNA resulting from cell lysis. In cases where the recombinant is the same size as the helper phage, it may be difficult to distinguish between the two species on a gel (M13KO7 is 8.7kb, R408 is 6.4kb, and pSELECT-Control is 5.6kb). In such cases, it may be advantageous to use the alternative helper phage supplied. The helper band is usually more prominent if R408 is used. The presence of the helper phage DNA does not interfere with the mutagenesis reaction.

D. Mutagenesis Reaction

The mutagenesis reaction involves annealing of the ampicillin repair oligonucleotide (provided) and the mutagenic oligonucleotide to the ssDNA template, followed by synthesis of the mutant strand with T4 DNA polymerase. The heteroduplex DNA is then transformed into the repair minus *E. coli* strain BMH 71-18 mut S. Mutants are selected by overnight growth in the presence of ampicillin. Plasmid DNA is then isolated and transformed into the JM109 strain. Mutant, ampicillin resistant colonies may be screened by direct sequencing of the plasmid DNA.

The amount of oligonucleotide required for the annealing reaction may vary depending on the size and amount of the single-stranded DNA template (pg. 100). The ampicillin repair oligonucleotide should be used at a 25:1 oligo:template ratio. A typical reaction may contain approximately 100ng (0.05pmol) of ssDNA.

Reagents to be Supplied by the User

Instructions for preparing buffers and media are provided on pg. 105.

- mutagenic oligonucleotide, phosphorylated (see phosphorylation reaction, pg. 100)
- sterile, deionized H_2O
- DMSO, frozen in aliquots
- competent cells (pg. 52) of the BMH 71-18 mut S and JM109 strains
- ampicillin
- LB medium
- LB plates + ampicillin
- mini-prep lysis buffer
- potassium acetate solution, pH 4.8
- 0.2N NaOH, 1% SDS (prepare fresh for each use)
- TE buffer
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- DNase-free RNase A (pg. 105)

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

Protocol

1. Prepare the mutagenesis or control annealing reactions as described below.

Mutagenesis Annealing Reaction

recombinant pSELECT-1 ssDNA	0.05pmol
ampicillin repair oligonucleotide (2.2ng/µl), phosphorylated	1µl (0.25pmol)
mutagenic oligonucleotide, phosphorylated (Table 10)	1.25pmol
annealing 10X buffer	2µl
sterile H_2O	to final volume 20µl

Control Annealing Reaction

pSELECT-Control ssDNA	100ng (0.05pmol)
ampicillin repair oligonucleotide (2.2ng/µl), phosphorylated	1µl (0.25pmol)
*lac*Z control oligonucleotide (10.8ng/µl), phosphorylated	1µl (1.25pmol)
annealing 10X buffer	2µl
sterile H_2O	to final volume 20µl

2. Heat the annealing reaction to 70°C for 5 minutes and allow it to cool slowly to room temperature (15-20 minutes).

Note: Single-stranded pSELECT-Control DNA must be prepared from the dsDNA provided.

3. Place the annealing reaction on ice and add the following:

synthesis 10X buffer	3µl
T4 DNA polymerase (10u/µl)	1µl
T4 DNA ligase (2u/µl)	1µl
sterile H_2O	5µl
	final volume will be 30µl

4. Incubate the reaction at 37°C for 90 minutes to perform mutant strand synthesis and ligation.

Table 10. Amount of Mutagenic Oligonucleotide Needed to Equal 1.25pmol.

Primer Length	ng of Primer Equal to 1.25pmol
17mer	7.0ng
20mer	8.3ng
23mer	9.5ng
26mer	10.8ng
29mer	12.0ng

Transformation into BMH 71-18 mut S

5. Add 3µl of DMSO to 200µl of BMH 71-18 mut S competent cells, mix briefly, and then add the entire synthesis reaction from Step 4. A procedure for preparing competent cells is provided on pg. 52. **Note:** DMSO should be stored frozen in aliquots and not reused once thawed.

6. Let the cells sit on ice for 30 minutes.

7. **Optional:** For some strains, a heat shock at 42°C for 1-2 minutes after the incubation on ice has been reported to increase transformation efficiency. In our experience, however, a heat shock does not significantly affect the efficiency of transforming BMH 71-18 mut S.

8. Add 4 ml of LB medium (pg. 105) and incubate at 37°C for 1 hour to allow the cells to recover.

9. Add ampicillin to a final concentration of 125µg/ml and incubate at 37°C for 12-14 hours with shaking.

Note: As a control to check the synthesis reaction, 1 ml of the culture may be removed after the one hour recovery step, spun down, resuspended in 50µl of LB medium, and plated on LB plates (pg. 105) containing 125µg/ml ampicillin. This is a check for the presence of ampicillin resistant transformants; a second round of transformation is necessary before screening for mutants.

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

E. Isolation and Screening of Mutant Colonies

pSELECT-1 or pSELECT-Control plasmid DNA is isolated from the overnight culture of BMH 71-18 mut S (Step 9, above) and transformed into the JM109 bacterial strain. Mutant colonies are screened by direct sequencing.

pSELECT Plasmid Mini-Prep Procedure

1. Place 1.5ml of the overnight culture into a microcentrifuge tube and centrifuge at 12,000 x g for 1 minute. The remainder of the overnight culture can be stored at 4°C.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Resuspend the pellet by vortexing in 100µl of ice-cold mini-prep lysis buffer (pg. 105).
4. Incubate for 5 minutes at room temperature.
5. Add 200µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS. Mix by inversion. **DO NOT VORTEX.** Incubate for 5 minutes on ice.
6. Add 150µl of ice-cold potassium acetate solution, pH 4.8 (pg. 105). Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.
7. Centrifuge at 12,000 x g for 5 minutes.
8. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
9. Add 1 volume of TE-saturated phenol/chloroform (pg. 105). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.
10. Transfer the upper, aqueous phage to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 9.
11. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of 100% ethanol. Mix and allow to precipitate 5 minutes on dry ice.
12. Centrifuge at 12,000 x g for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.
13. Dissolve the pellet in 50µl of sterile deionized water. Add 0.5µl of 100µg/ml DNase-free RNase A (pg. 105) and incubate for 5 minutes at room temperature.
14. The yield of plasmid DNA can be determined by electrophoresis on an agarose gel. A yield of 1-3µg of plasmid DNA may be expected.

Transformation into JM109

15. Add 3µl of DMSO to 200µl of JM109 competent cells, mix briefly, and add 0.05-0.10µg of plasmid DNA from Step 14. JM109 competent cells may be purchased from Promega or prepared using the procedure provided on pg. 52.
16. Let the cells sit on ice for 30 minutes.
17. **Optional:** A heat shock may be performed at this step. See pg. 53, Step D.7.
18. Add 2 ml of LB medium and incubate at 37°C for 1 hour to allow the cells to recover.
19. Divide the culture into two microcentrifuge tubes and spin for 1 minute in a microcentrifuge.
20. Pour off the supernatant and resuspend the cells in each tube in 50µl of LB medium.
21. Plate the cells in each tube on an LB plate containing 125µg/ml ampicillin and incubate at 37°C for 12-14 hours.

Analysis of Transformants

The Altered Sites mutagenesis procedure generally produces greater than 50% mutants, so colonies may be screened by direct sequencing. Assuming that greater than 50% mutants are obtained, screening 5 colonies will give a greater than 95% chance of finding the mutation. If the mutation is located within 200-300 bases of either end of the DNA insert, the SP6 or T7 sequencing primers may be used for convenient priming of the sequencing reactions.

VII. Site-Directed *in vitro* Mutagenesis with the Altered Sites™ System

(continued)

Solutions Required

Kinase 10X buffer:

500mM	Tris-HCl, pH 7.5
100mM	$MgCl_2$
50mM	DTT
1.0mM	spermidine
10mm	ATP

TYP broth (per liter):

16g	Bacto-tryptone
16g	Bacto-yeast extract
5g	NaCl
2.5g	K_2HPO_4

Autoclave and cool. Where indicated, add tetracycline to 15µg/ml.

Phage precipitation solution:

3.75M	ammonium acetate, pH 7.5
20%	polyethylene glycol (MW 8,000)

Add equal volumes of 40% PEG-8,000 stock solution and 7.5M ammonium acetate, pH 7.5.

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol, phase with 1 part of chloroform:isoamyl alcohol (24:1).

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust pH to 7.5 with NaOH and autoclave.

LB plates plus ampicillin (per liter):

Add 15g agar to 1 liter of LB medium. Adjust to pH 7.0 with NaOH. Autoclave. Allow the medium to cool to 55°C before adding ampicillin (125µg/ml final conc.). Pour 30-35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

Annealing 10X buffer:

200mM	Tris-HCl pH 7.5
100mM	$MgCl_2$
500mM	NaCl

Synthesis 10X buffer:

100mM	Tris-HCl pH 7.5
5mM	dNTPs
10mM	ATP
20mM	DTT

Mini-prep lysis buffer*:

25mM	Tris-HCl, pH 8.0
10mM	EDTA
50mM	glucose

Potassium acetate solution, pH 4.8:

Prepare 60ml of 5M potassium acetate. Add 11.5ml of glacial acetic acid and 28.5ml of H_2O. This solution is 3M with respect to potassium and 5M with respect to acetate. Store at 4°C.

DNase-free RNase A:

To make DNase-free RNase A, prepare a 10mg/ml solution of RNase A in 10mM Tris-HCl, pH 7.5, 15mM NaCl. Heat at 100°C for 15 minutes and cool to room temperature.

*Buffer tablets available from Promega.

Nucleic Acid Sequencing and Mutagenesis

VIII. Double-Stranded DNA Template Preparation

After transforming into *E. coli* host strains and plating on selective media, cells harboring recombinant plasmid DNA will grow into colonies. Individual colonies are grown and the cultured cells are processed to produce double-stranded template for the sequencing reaction. Two protocols are provided for isolation of plasmid DNA: a large-scale procedure (Section A) yielding about 500µg DNA and a mini-prep procedure (Section B) yielding 1-4µg DNA. Protocols for the isolation of lambda DNA are provided in the Nucleic Acid Detection, Purification, and Labeling chapter (pg. 136).

Plasmid Prep Reagents Needed

Instructions for preparing these solutions are provided on pg. 108.

- LB medium
- ampicillin
- 0.2N NaOH, 1% SDS
- DNase-free RNase A
- TE buffer
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- ethanol (100% and 70%)
- 4M NaCl
- 13% PEG

For large-scale plasmid prep (Section A):

- lysis buffer
- 3M sodium acetate, pH 4.6

For plasmid mini-prep (Section B):

- mini-prep lysis buffer
- potassium acetate solution, pH 4.8

A. Rapid Isolation of Plasmid DNA

This protocol was obtained from Drs. Paul Kreig and Doug Melton, Harvard University.

1. Pick a single colony from a plate and inoculate 250ml of LB medium containing 50µg/ml ampicillin. Grow the cells overnight in a shaker at 37°C.

2. Centrifuge the cells at 5,000 x g, 4°C for 15 minutes.

3. Resuspend the cell pellet in 6ml of ice-cold freshly prepared lysis buffer (pg. 108). Thoroughly resuspend the cells by pipetting them up and down with a 10ml pipette. Incubate in ice water for 10 minutes.

4. Add 12ml of freshly prepared 0.2N NaOH and 1% SDS. Carefully mix by inversion and incubate in ice water for 10 minutes. **DO NOT VORTEX.**

5. Add 7.5ml of 3M sodium acetate, pH 4.6. Carefully mix by inversion or gentle vortexing and incubate in ice water for 20 minutes.

6. Centrifuge at 12,000 x g for 15 minutes. Transfer the supernatant to another tube and discard the precipitate.

7. Add DNase-free RNase A (see pg. 108 for preparation) to a final concentration of 20µg/ml. Incubate at 37°C for 20 minutes.

8. Extract twice with 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

9. Transfer the upper, aqueous phase to a fresh tube and add 1 volume chloroform:isoamyl alcohol (24:1), vortex for 1 minute, and centrifuge as in Step 8.

10. Transfer the upper, aqueous phase to a fresh tube. Add two volumes of ethanol and leave at –20°C for 30 minutes. Centrifuge at 12,000 x g for 20 minutes.

11. OPTIONAL: Carefully drain the tube and dissolve the DNA pellet in 1.6ml of H_2O. Add 0.4ml of 4M NaCl and mix. Add 2ml of 13% polyethylene glycol (PEG) and mix. Incubate in ice water 60 minutes. A yield of approximately 500µg DNA should be expected. Centrifuge at 12,000 x g for 10 minutes.

12. Remove the supernatant and wash the pellet with 5ml of 70% ethanol. Centrifuge at 12,000 x g for 5 minutes.

13. Dry the pellet under vacuum and dissolve the DNA in an appropriate volume of water or TE buffer (100-500µl).

VIII. Double-Stranded DNA Template Preparation

(continued)

B. Plasmid Mini-Prep Procedure

1. Inoculate 5ml of LB medium (pg. 108) containing 50µg/ml ampicillin with a single bacterial colony. Incubate at 37°C overnight with vigorous shaking.

2. Place 1.5ml of culture into a microcentrifuge tube and centrifuge at 12,000 x g for 1 minute. The remainder of the overnight culture can be stored at 4°C.

3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

4. Resuspend the pellet by vortexing in 100µl of ice-cold lysis buffer (pg. 108).

5. Incubate for 5 minutes at room temperature.

6. Add 200µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS. Mix by inversion. **DO NOT VORTEX.** Incubate for 5 minutes on ice.

7. Add 150µl of ice-cold potassium acetate, pH 4.8 (pg. 108). Mix by inversion or gently vortexing for 10 seconds. Incubate on ice for 5 minutes.

8. Centrifuge at 12,000 x g for 5 minutes.

9. Transfer the supernatant to a fresh tube, avoiding the white precipitate.

10. Add DNase-free RNase A (see pg. 108 for preparation) to a final concentration of 20µg/ml.

11. Incubate at 37°C for 20 minutes.

12. Add 1 volume of TE-saturated phenol/chloroform (pg. 108). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

13. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 13.

14. Transfer the upper, aqueous phase to a fresh tube and add 2.5 volumes of ethanol. Mix and allow to precipitate 5 minutes on dry ice.

15. Centrifuge at 12,000 x g for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.

16. Dissolve the dried pellet in 10µl of sterile deionized water. A yield of 1-4µg DNA should be expected from 1.5ml of culture (Step B.2).

Alkali Denaturation Reagents Needed

- 2M NaOH, 2mM EDTA
- 2M ammonium acetate, pH 4.6
- ethanol (100% and 70%)

C. Alkali Denaturation of Supercoiled Plasmid DNA

To prime efficiently, double-stranded plasmids must be converted to a single-stranded form prior to sequencing. This is accomplished by alkali denaturation of supercoiled plasmid DNA.

1. Pipet a volume containing 4µg (approximately 2pmol) of supercoiled plasmid DNA in a microcentrifuge tube and add deionized H_2O to 18µl.

2. Add 2µl of a 2M NaOH, 2mM EDTA solution and incubate for 5 minutes at room temperature.

3. Neutralize the reaction with 2µl of 2M ammonium acetate, pH 4.6, and vortex to mix.

4. Add 75µl of ethanol and vortex. Place on dry ice (or at –70°C) for 10 minutes.

5. Centrifuge the tube for 10 minutes at top speed in a microcentrifuge.

6. Decant the supernatant. Wash with 200µl of cold 70% ethanol and centrifuge for 1 minute.

7. Remove the supernatant and dry the pellet. Resuspend the dried pellet in 5µl distilled water for sequencing with Klenow or RT, or in 18µl distilled water for sequencing with *Taq* DNA polymerase. Proceed to the annealing step.

VIII. Double-Stranded DNA Template Preparation

(continued)

D. Alkali Denaturation of Lambda DNA

Note: Lambda DNA should be sequenced with the TaqTrack® system using an end-labeled primer.

1. Pipet a volume containing 10µg (approximately 0.4pmol) of phage lambda DNA in a microcentrifuge tube and add distilled H_2O to 100µl.
2. Add 10µl of a 2M NaOH, 2mM EDTA solution and incubate for 5 minutes at room temperature.
3. Neutralize the reaction with 10µl of 2M ammonium acetate, pH 4.6, and vortex to mix.
4. Add 375µl of ethanol and vortex. Let set on dry ice (or at –70°C) for 10 minutes.
5. Centrifuge for 10 minutes in a microcentrifuge.
6. Decant the supernatant. Wash with 200µl of cold 70% ethanol and centrifuge for 5 minutes.
7. Remove the supernatant and dry the pellet. Resuspend the dried pellet in 19µl distilled water (for sequencing with *Taq* DNA polymerase) and proceed to the annealing step.

Composition of Solutions

Lysis buffer:

25mM	Tris-HCl, pH 8.0
10mM	EDTA
50mM	glucose

TE buffer*:

10mM	Tris-HCl, pH 7.5-8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

DNase-free RNase A:

To make DNase-free RNase A, prepare a 10mg/ml solution of RNase A in 10mM Tris-HCl, pH 7.5, 15mM NaCl. Heat at 100°C for 15 minutes and cool slowly to room temperature.

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto yeast Extract
5g	NaCl

Adjust pH to 7.5 with NaCl and autoclave. Where indicated, add ampicillin to 50µg/ml.

Potassium acetate solution, pH 4.8:

Prepare 60ml of 5M potassium acetate. Add 11.5ml of glacial acetic acid and 28.5ml of H_2O. This solution will be 3M with respect to potassium and 5M with respect to acetate. Store at 4°C.

*Buffer tablets available from Promega.

IX. Single-Stranded DNA Template Preparation from M13 Vectors or pGEM®-Zf Phagemids

A. Preparation of M13 Single-Stranded Template

After transforming into appropriate *E. coli* host strains and plating on indicator media, cells containing M13 recombinants will appear as colorless "plaques" (31). These infected cells are not lysed or killed by the phage. The clear areas represent retarded growth of the *E. coli* which appears as a plaque when surrounded by faster growing uninfected cells. Infected cells from such colorless "plaques" are grown up to produce single-stranded template for the sequencing reaction.

Reagents Needed

Instructions for preparing these reagents are supplied on pg. 113.

- TYP broth
- phage precipitation solution
- TE buffer
- chloroform:isoamyl alcohol (24:1)
- TE-saturated phenol/chloroform
- 7.5M ammonium acetate, pH 7.5
- ethanol (100% and 70%)

Protocol

1. Dilute an overnight culture of host cells (e.g., NM522 or JM101) 1:100 in 3ml of TYP broth and infect with a single plaque of recombinant M13.
2. Shake vigorously for 6-8 hours at 37°C.
3. Harvest the supernatant (use the entire 3ml) by pelleting the cells at 12,000 x g for 15 minutes in two 1.5ml microcentrifuge tubes. Pour the supernatant into a fresh tube and spin again for 15 minutes.
4. Precipitate the phage by adding 0.25 volume of phage precipitation solution to the supernatants. Set on ice for 30 minutes, then centrifuge for 15 minutes at 12,000 x g. Thoroughly drain the supernatants.
5. Resuspend both pellets in 400µl of TE buffer.
6. Add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 full minute and centrifuge at 12,000 x g for 5 minutes.
7. Transfer the upper, aqueous phase to a fresh tube, leaving the large interface behind. Add 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and centrifuge as in Step 6.
8. Transfer the upper, aqueous phase to a fresh tube and repeat the phenol extraction as in Step 7. It may be necessary to repeat the extractions several times, until there is no visible material at the interface.
9. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume of 7.5M ammonium acetate, pH 7.5, and 2 volumes of 100% ethanol. Mix and leave at –20°C for 30 minutes.
10. Centrifuge at 12,000 x g for 15 minutes, remove the supernatant, and carefully rinse the pellet with 70% ethanol. If the pellet is disturbed, respin. Drain the tube and dry the pellet under vacuum.
11. Resuspend the DNA pellet in 20µl of deionized water. The amount of DNA present can be estimated by agarose gel electrophoresis of a 2µl sample. If sufficient DNA is present, proceed to the appropriate annealing step (in Section II, III or IV, pgs. 80-85).

B. General Considerations for Use of pGEM®-Zf Phagemids

The pGEM single strand systems are designed to allow the production of cloned DNA as single-stranded circular molecules suitable for mutagenesis, sequencing, and other applications (25-27). Single-stranded DNA can be produced from any of the pGEM-Zf(+) and pGEM-Zf(-) series of plasmids, which contain the origin of replication of the filamentous bacteriophage f1. The orientation of the f1 DNA, designated by (+) or (-), determines which of the two strands of the plasmid will be secreted. The plasmids also contain SP6 and T7 RNA polymerase promoters flanking a region of multiple cloning sites within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates. For induction of single-stranded DNA (ssDNA), bacterial cells containing pGEM-Zf recombinants are infected with an appropriate helper phage. The plasmid then enters the f1 replication mode and the resulting

Nucleic Acid Sequencing and Mutagenesis

IX. Single-Stranded DNA Template Preparation from M13 Vectors or pGEM®-Zf Phagemids

(continued)

ssDNA is exported from the cell as an encapsidated virus-like particle. The single-stranded plasmid DNA is purified from the supernatant by simple precipitation and extraction procedures.

These chimeric ssDNA phage-plasmid vectors, or phagemids, share many of the properties of the filamentous bacteriophages from which they are derived (32). Thus, the protocols and precautions involved in using these biological tools are similar. A number of procedural details concerning filamentous phage vectors that also apply to using the pGEM single strand system can be found in reference 31. Single-stranded DNA template can be prepared from any desired volume of culture, depending on the amount needed and productivity of the particular plasmid/host/helper phage combination. The following protocol is intended for processing 5ml of culture; proportional amounts of the various reagents should be used with other volumes.

C. Preparation of Single-Stranded Phagemid DNA

Reagents Needed

(See list of reagents needed for preparing M13 templates, pg. 109.)

Protocol

1. Prepare an overnight culture of cells containing pGEM®-Zf phagemid DNA by picking individual ampicillin resistant colonies from a fresh plate. Inoculate 1-2ml of TYP broth containing 50µg/ml ampicillin and shake at 37°C.

2. The next morning, inoculate 5ml of TYP broth with 100µl of the overnight culture. Shake vigorously at 37°C for 30 minutes in a 50ml tube.

3. Infect with helper phage R408 or M13KO7 at an m.o.i. (multiplicity of infection) of 10 (i.e., add 10 helper phage particles per cell). For the helper phages supplied with this system, add 40µl (see Note 4). Continue shaking for 6 hours to overnight with vigorous agitation and good aeration.

4. Harvest the culture supernatant by pelleting the cells at 12,000 x g for 15 minutes. Pour the supernatant into a fresh tube and spin again for 15 minutes (see Notes 5 and 6).

5. Precipitate the phage by adding 0.25 volume of phage precipitation solution to the supernatant. Leave sitting on ice for 30 minutes, then centrifuge for 15 minutes at 12,000 x g. Thoroughly drain the supernatant.

6. Resuspend the pellet in 400µl of TE buffer and transfer the sample to a microcentrifuge tube.

7. Add 0.4ml of chloroform:isoamyl alcohol (24:1) to lyse the phage, vortex for 1 full minute, and centrifuge at 12,000 x g for 5 minutes. This step removes excess PEG.

8. Transfer the upper, aqueous phase to a fresh tube, leaving the large interface behind. Add 0.4ml of TE-saturated phenol/chloroform, vortex for 1 full minute and centrifuge as in Step 7.

9. Transfer the upper, aqueous phase to a fresh tube and repeat the phenol extraction as in Step 8. It may be necessary to repeat the extractions several times until there is no visible material at the interface.

10. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume (200µl) of 7.5M ammonium acetate and 2 volumes (1.2ml) of ethanol. Mix and leave at –20°C for 30 minutes to precipitate the phagemid DNA.

11. Centrifuge at 12,000 x g for 15 minutes, remove the supernatant, carefully rinse the pellet with 70% ethanol and spin for 2 minutes. Drain the tube and dry the pellet under vacuum. The pellet may be difficult to see.

12. Resuspend the DNA in 20µl of H_2O. The amount of DNA present can be estimated by agarose gel electrophoresis of a 2µl sample.

Notes:

1. Two factors that may improve the yield of ssDNA are the use of an enriched broth, such as TYP, and vigorous aeration. The use of a smaller test tube than that suggested in step 2 for 5ml preps will diminish the yield. To obtain optimal aeration of large volumes, use a flat bottom flask containing 0.2 volume of the culture and shake at high RPM.

2. The presence of ampicillin, at least up to 100µg/ml, in the culture media in Step 2 does not seem to alter the yield of ssDNA obtained.

IX. Single-Stranded DNA Template Preparation from M13 Vectors or pGEM®-Zf Phagemids

(continued)

3. The amount of time between initial cell inoculation and infection with helper phage (Steps 2 and 3) is not critical to obtaining high yields of ssDNA. Helper phage infection as late as 3 hours after starting the cell cultures has resulted in the same overall yield as early infection.

4. Adding 40µl of a helper phage stock at 5×10^{10} pfu/ml gives an m.o.i. (multiplicity of infection) of approximately 10. An m.o.i. as high as 100 will not alter the yield of ssDNA.

5. Pelleting the cells twice (Step 4) is important for reducing the level of contaminating cellular nucleic acid in the supernatant.

6. An additional level of purity of ssDNA can be attained by treating the supernatant (after Step 4 but before Step 5) with DNase I (10u/ml plus 2mM $MgCl_2$) and RNase A (10µg/ml) for 15 minutes at 37°C.

D. Analysis of Single-Stranded DNA Purity

The purity of DNA is accurately determined by the A_{260}/A_{280} ratio. Because a highly purified DNA template is required for successful dideoxy sequencing and oligonucleotide mutagenesis, the absorbance ratio should be greater than 1.8. If not, repeat the phenol:chloroform extractions. In very clean preparations, the A_{260} is a good estimate of single-stranded plasmid concentration. A 1mg/ml solution of ssDNA has an A_{260} of 33.

The amount and purity of single-stranded plasmid DNA can be estimated by agarose gel electrophoresis and ethidium bromide staining. Two major bands are usually seen on 1% gels run in Tris-acetate buffer: the helper phage DNA and the single-stranded plasmid. There may also be a small amount of large chromosomal DNA and possibly some RNA that is the result of cell lysis in some preparations. In cases where the recombinant is the same size as the helper phage, it may be difficult to distinguish between the two species on a gel (M13KO7 is 8.7kb and R408 is 6.4kb). The helper band is usually more prominent if R408 is used. The presence of the helper phage DNA has not interfered with sequencing or oligonucleotide mutagenesis procedures. The supercoiled pGEM-Zf vector DNAs comigrate with M13KO7 helper phage DNA in gels run at 3 V/cm. Thus, the nonrecombinant double-stranded plasmids can serve as approximate markers for this DNA.

E. Preparation of Helper Phage Stocks

The pGEM-Zf plasmids require the presence of a helper phage in order to be propagated, packaged, and secreted as ssDNA viral particles. M13KO7 (33) and R408 (34) are helper phages which allow the preferential secretion of single-stranded plasmid DNA over the phage ssDNA, thereby increasing the absolute yield of plasmid ssDNA. Both helper phages are offered as part of this system in order to provide the greatest latitude in optimizing ssDNA yields. Depending on the host and the particular vector combination, differences in yields and absolute amounts of plasmid and phage ssDNA have been observed with the two helper phages. Although no rules have emerged as to which one to use under which circumstances, in general both helper phages perform well for the intended purpose.

The procedures for growth of the two helper phages are somewhat different because of inherent differences between them. M13KO7 has a kanamycin resistance marker that is spontaneously deleted with high frequency in the absence of antibiotic selection, so a single kanamycin resistant colony must be used to propagate the virus. In contrast, R408 can be propagated from a single plaque on media without antibiotic selection.

Reagents Needed

- TYP Broth (pg. 113)
- kanamycin
- B broth, top agar and plates (pg. 113)

Protocol

1. Growth of Phage M13KO7

a. Make a total of five 1/100X serial dilutions of the supplied phage stock in TYP broth (pg. 113). Begin by adding 0.1ml of phage to 9.9ml of TYP broth.

b. In separate sterile tubes, add 0.1ml of each dilution to 0.2ml of log phase NM522 or JM109 cells (1ml of cells grown overnight and then added to 10ml TYP broth and shaken for 60 minutes). Incubate the tubes at room temperature for 5 minutes.

c. Plate 50µl of each cell and phage mixture on TYP plates containing 70µg/ml kanamycin. Invert the plates and incubate overnight at 37°C.

Nucleic Acid Sequencing and Mutagenesis

IX. Single-Stranded DNA Template Preparation from M13 Vectors or pGEM®-Zf Phagemids

(continued)

d. Pick a single, well-isolated plaque with a sterile Pasteur pipet and inoculate 50-250ml of TYP broth containing 70µg/ml of kanamycin. Incubate the culture at 37°C for 8-12 hours with vigorous agitation.

e. Pellet the cells by centrifugation at 12,000 x g for 15 minutes in autoclaved tubes. Pour the supernatant (containing phage) into a fresh tube and spin again for 15 minutes to remove additional cells.

f. Pour the supernatant into a sterile storage container and heat at 55°C for 30 minutes to kill any remaining cells. The phage in the supernatant will remain viable for several years when stored at 4°C.

2. Growth of Phage R408

a. Make a total of five 1/100X serial dilutions of the supplied phage stock in B broth (pg. 113). Begin by adding 0.1ml of phage stock to 9.9ml of B broth.

b. In separate sterile tubes, add 0.1ml of each dilution to 0.2ml of log phase NM522 or JM109 cells (1ml of cells grown overnight and then added to 10ml TYP broth and shaken for 60 minutes). Incubate the tubes at room temperature for 5 minutes.

c. Add 4ml of B top agar (45°C) and pour the cell/phage mixture on B plates. Invert the plates and incubate them overnight at 37°C.

d. Pick a single, well-isolated plaque and use it to inoculate 50-250ml of TYP broth. Incubate the culture at 37°C for 8-12 hours with vigorous agitation.

e. Pellet the cells by centrifugation at 12,000 x g for 15 minutes in autoclaved tubes. Pour the supernatant into a fresh tube and spin it again for 15 minutes to remove additional cells.

f. Pour the supernatant into a sterile storage container and heat it at 55°C for 30 minutes to kill any remaining cells. The phage in the supernatant will remain viable for several years when stored at 4°C.

3. Titering Phage M13KO7 and R408

a. Serially dilute the desired phage stock, mix it with log phase NM522 or JM109 cells, and plate it on B plates as described in Steps 2.a-2.c, above.

b. Count and calculate the number of plaque forming units/ml (pfu/ml). **Example:** If there are 3 plaques on the 10^{10} dilution plate (dilution number 5), there are $3 \times 10^{10} \times 10 = 3 \times 10^{11}$ pfu/ml. The last factor of 10 in the calculation converts the 0.1ml plated to a per milliliter basis.

F. Troubleshooting Single-Stranded DNA Yields

In general, obtaining plasmid sequences as ssDNA with this system is identical to the procedures necessary for obtaining M13, f1, fd, or IKE phage as ssDNA. However, the yield of single-stranded plasmid DNA obtained will usually be lower than that observed with the ssDNA bacteriophages. This is due to the inherent nature of the helper phage infection procedure and the interference of the mini-phage or plasmid. Therefore, visualization of ssDNA on an agarose gel of an aliquot prepared directly from the supernatant, as is done with M13 ssDNA, may be difficult. It may be necessary to concentrate the DNA first by PEG precipitation (pg. 110, Steps 5-7). Because of the large amount of debris in the PEG pellet, the DNA should be chloroform-extracted (pg. 110, Step 7) before it is run on a gel.

Previous experience with M13 procedures should help to identify potential problems. The helper phages R408 or M13KO7 should be purified from a control supernatant to assist in identifying the appropriate ssDNA bands on an agarose gel. To do this, simply infect cells without a plasmid and process these along side the ssDNA plasmid samples.

To obtain any ssDNA or phage, the strain you are working with has to have an F factor (episome). The selection for this with JM109 and NM522 is to maintain a stock on minimal (M9) plates without amino acids (31). Always use this stock to prepare competent cells for transformation. Serial passage of the strains may result in the rapid loss of the pilus, which is required for infection by the virus. After transforming cells and growing them on ampicillin plates, keep them stored at 4°C. If you need the

Nucleic Acid Sequencing and Mutagenesis

IX. Single-Stranded DNA Template Preparation from M13 Vectors or pGEM®-Zf Phagemids

(continued)

cells for longer than 1 month, store them as glycerol stocks at –70°C. Do not use cells that have been at room temperature for more than several hours to obtain ssDNA. Using freshly plated cells and storing cells in the cold as soon as the plates come out of the incubator seems to be important for obtaining good yields of ssDNA.

It will be helpful in determining where problems are occurring if ssDNA is isolated from cells transformed with a nonrecombinant pGEM®-Zf plasmid. If you cannot obtain ssDNA from this control, streak cells containing the plasmid onto minimal (M9) plates and allow several days for colonies to come up. If nothing grows, the episome has been lost. Another reason for performing the empty vector control is that the 3.2kb single-stranded plasmid DNA should be clearly visible and distinct from the 6.4kb (R408) or 8.7kb (M13KO7) helper phage DNA on an agarose gel. The presence of minor amounts of high molecular weight chromosomal DNA should not interfere with sequencing.

If you follow the above precautions and the yields of ssDNA are still poor, try growing larger amounts or growing cultures for longer periods of time. However, prolonged growth after infection may result in significant contamination of the ssDNA with chromosomal DNA, presumably due to cell death and lysis in the late stationary phase. Certain sequences or a particular orientation of DNA may influence the copy number of the plasmid, thereby decreasing the yield of ssDNA. Also, a particular sequence or orientation of DNA may be extremely unstable to ssDNA replication.

Solutions Required

TYP broth (per liter):

16g	Bacto-tryptone
16g	Bacto-yeast extract
5g	NaCl
2.5g	K_2HPO_4

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Phage precipitation solution:

3.75M	ammonium acetate, pH 7.5.
20%	polyethylene glycol (MW 8,000)

Add equal volumes of 40% PEG-8000 stock solution and 7.5M ammonium acetate, pH 7.5.

B broth (per liter):

10g	Bacto-tryptone
8g	NaCl

After autoclaving and cooling, add 10ml of sterile 20% glucose.

B top agar:

B broth plus 6g Bacto-agar per liter

After autoclaving and cooling, add 10ml of sterile 20% glucose.

B plates:

B broth plus 15g Bacto-agar per liter

After autoclaving and cooling, add 10ml of sterile 20% glucose.

*Buffer tablets available from Promega.

Nucleic Acid Sequencing and Mutagenesis

X. General Considerations for Sequencing Gels

The DNA products of sequencing reactions separate in polyacrylamide gels as a function of the log of their molecular weight. As a result, the distance between smaller fragments is greater than that separating larger fragments. The rate of migration of DNA fragments in the gel is a function primarily of the voltage gradient (volts/cm of gel length). Thus, longer gels require a greater voltage to achieve a given speed of separation. The amount of heat generated during the run is dependent on the current (amps) used. A good strategy is to run gels at constant power rather than constant voltage or current. Since power is the product of voltage and current (watts = volts x amps), this prevents large voltage spikes or excessive heating from occurring. The power is typically adjusted to maintain the run temperature at 50-60°C, which is very warm or hot to the touch. This temperature is not enough to keep DNA fragments denatured without cracking the gel plates.

Preparation of the Gel

It is essential to use only ultrapure reagents in preparing DNA sequencing gels. Promega's Acryl-a-Mix™ 6 and Acryl-a-Mix™ 8 pre-mixed sequencing solutions are convenient alternatives to preparing your own 6% and 8% gel solutions. The researcher need only add 10% ammonium persulfate to the pre-mixed acrylamide solution and pour the gel directly from the bottle. Table 11, below, provides recipes for a range of sequencing gels for those who prefer to prepare their own solutions. Lower percentage polyacrylamide gels will allow fragments to run faster, but do not necessarily provide greater resolution of fragments longer than 100 base pairs. The sizes of DNA fragments comigrating with the bromophenol blue and xylene cyanol tracking dyes in various percentage polyacrylamide gels are listed in Table 12.

The rate of polymerization of the gel is a function of the acrylamide concentration, the catalyst concentration and the ambient temperature. The TEMED and ammonium persulfate catalysts should be thoroughly mixed with the acrylamide solution to ensure homogeneous polymerization, and gels should be allowed to polymerize for at least 1 hour. Polymerization is inhibited by O_2.

Table 11. Components of Sequencing Gel Solutions.

Gel %	Acrylamide	Bis-Acrylamide	Urea	TBE'10X Buffer (pg. xx)	Deionized H_2O	10%AP*	TEMED
6%	5.7g	0.3g	48g	10ml	40ml	500µl	50µl
8%	7.6g	0.4g	48g	10ml	40ml	500µl	50µl
10%	9.5g	0.5g	48g	10ml	40ml	500µl	50µl
12%	11.4g	0.6g	48g	10ml	40ml	500µl	50µl
16%	15.2g	0.8g	48g	10ml	40ml	500µl	50µl
20%	19.0g	1.0g	48g	10ml	40ml	500µl	50µl

*10% ammonium persulfate should be made fresh weekly in deionized H_2O and stored at room temperature.

X. General Considerations for Sequencing Gels

(continued)

A more uniform spacing of gel bands can be achieved by using wedge-shaped gel spacers (slightly wider at the bottom) or by pouring buffer gradient gels (higher salt concentration towards the bottom). The effect of both of these techniques is to alter the voltage gradient in the gel such that smaller bands are slowed down. As a result, more sequence information can be read from a single autoradiogram.

Because of the high urea concentration in a sequencing gel, urea will tend to diffuse into the sample wells and, because of its high density, interfere with sample loading. Sample wells should be flushed with gel buffer just before the samples are loaded.

Table 12. Migration of Tracking Dyes in Denaturing Polyacrylamide Gels.

Gel %	Comigrating DNA	Fragment Size
	Bromophenol Blue	Xylene Cyanol
6%	26bp	106bp
8%	19bp	70-80bp
10%	12bp	55bp
20%	8bp	28bp

Running the Gel

A number of factors can cause anomalous migration of DNA fragments in sequencing gels. Since the temperature of the gel affects its conductivity, the uniformity of the voltage gradient and thus the mobility of bands is dependent on uniform heat distribution. This can be achieved with a circulating buffer chamber or by placing an aluminum plate in contact with the gel plate. If a detachable aluminum plate is used, it should be placed behind the gel to minimize the risk of electrical shock.

Thickness variations in the gel can also cause aberrant migration due to variations in the voltage gradient. Use only high quality glass plates (not window glass) and do not clamp the sides of the gel plates when pouring the gel. Use tape to seal the sides and clamps only at the bottom. Be sure to support the gel uniformly to prevent bowing of the plates.

Excessive salt in the sample also can lead to a variety of problems, including slower migration, lanes with a pinched appearance or a significantly arched dye front across the gel.

Solution Composition

TBE 10X buffer:

0.89M Tris-base, pH 8.0
0.89M boric acid
20mM EDTA

Nucleic Acid Sequencing and Mutagenesis

XI. Troubleshooting Sequencing Reactions

Symptoms	Possible Causes	Comments
Faint bands or no bands, even when known DNA template is used.	Insufficient or dirty template DNA.	Prepare new template DNA or use a larger amount of DNA.
	Insufficient enzyme activity.	Use a fresh preparation of enzyme or add more enzyme, up to 4X the recommended level.
	Isotope too old.	Use fresh isotope. ^{32}P should be used within 2 weeks. ^{35}S can be used as long as 2 months if stored at –70°C.
	Poor annealing of primer to template.	Verify that the primer sequence is correct for the template DNA.
		Make sure the primer does not self-anneal or form hairpin structures.
	Contamination of sequencing reaction with protein or salt.	If possible, check the A_{260}/A_{280} ratio. This should be 1.8-2.0. If lower, re-extract with phenol. Excess salt can be removed by reprecipitating with ethanol and then washing the pellet with 70% ethanol before drying.
	Samples not denatured before loading on gel.	Make sure samples are properly heat denatured.
Low band intensity at bottom of gel.	When using a two-step extension/labeling protocol, bands at the bottom of the gel are inherently fainter because shorter fragments have incorporated less isotope. Several procedural modifications can lead to increased termination and thus darker bands closer to the primer.	Raise the DNA concentration 2 to 3-fold.
		Check that the labeling reaction contains the correct dNTP concentrations.
		Reduce the concentration of the dNTP extension mix 2-fold in the labeling reaction.
		Reduce the labeling reaction time to 1-2 minutes.
	Ratio of ddNTPs to dNTPs is too low.	Make up fresh ddNTP mixes or increase the ratio of ddNTPs to dNTPs 2 to 4-fold.
Low band intensity at top of gel or limited length of read.	Ratio of ddNTP to dNTP is too high.	Make up fresh ddNTP mixes or decrease the ratio of ddNTPs to dNTPs 2- to 4-fold in the termination reaction.

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XI. Troubleshooting Sequencing Reactions

(continued)

Symptoms	Possible Causes	Comments
Short read length or faint bands, occurring in isolated lanes.	When short read length occurs only in isolated lanes, premature termination may be caused by poor pipetting or mixing.	Use only high quality pipet tips, mix well at all steps, and spin tubes briefly after adding reagents to ensure that no liquid remains on the tube walls.
	If problems occur consistently with one nucleotide mix, the mix may be contaminated or the mix may be poorly balanced.	Prepare fresh nucleotide mixes.
		Adjust the ddNTP and dNTP concentrations as described above.
High background in each lane or a smear of uniform intensity down each lane.	Contamination of template with RNA.	Prepare new template.
	Contamination of template with PEG.	Prepare new template.
	Problem with isotope.	Use fresh isotope. ^{32}P should be used within 2 weeks. ^{35}S can be used as long as 2 months if stored at –70°C.
Bands are fuzzy throughout the lanes.	Dirty template DNA.	Prepare new template DNA.
Poor quality polyacrylamide gel.	Prepare fresh acrylamide and buffer solutions using high-quality reagents.	Store solutions at 4°C in the dark.
	Electrophoresis temperature too high.	Run gel at lower temperature (40-60°C).
Bands are fuzzy in certain areas of gel.	Poor contact of film with gel.	Make sure film is clamped tightly to gel.
	Wrinkle in dried gel.	Be very careful to avoid wrinkles when drying gel.
Bands at the same position in two or three lanes, occurring throughout the gel.	DNA sample contains two different templates, generating overlapping sequences.	Prepare new template DNA, starting with a single plaque or colony.
	Primer hybridizing to a secondary site.	Increase stringency of annealing or make a new primer.
	Priming occurring at nicks or gaps in DNA template or at contaminating DNA fragments.	Use an end-labeled primer. DNA chains extended from nicks, gaps or contaminating fragments will not be labeled.
	When non-sequencing grade *Taq* DNA polymerase is used to sequence double-stranded templates using the two-step extension/labeling protocol, residual 5′→3′ exonuclease activity can lead to ghost bands.	Use Promega's sequencing grade *Taq* DNA polymerase, which has been specifically modified to remove 5′→3′ exonuclease activity.

(continued on next page)

Nucleic Acid Sequencing and Mutagenesis

XI. Troubleshooting Sequencing Reactions

(continued)

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Symptoms	Possible Causes	Comments
Bands in the same position in all four lanes, occurring throughout the gel.	Insufficient enzyme activity.	Use a fresh preparation of enzyme or add more enzyme, up to 4X the recommended level. Promega's T7 DNA polymerase is less prone to strong stops than other preparations of this enzyme.
	Dirty template DNA.	Prepare new template DNA.
	DNA template is nicked or contaminated with PEG.	Remove nicked DNA by acid-phenol extraction (pg. 91). Remove excess PEG by reprecipitating with ethanol. Resuspend pellet in 10mM Tris-HCl, pH 7.6, and extract with chloroform, then ethanol precipitate the aqueous phase.
Anomolous spacing of bands, missing bands, and bands at the same position in two or three lanes, occurring only at specific regions.	Band compression: a newly synthesized DNA strand is forming secondary structure during gel electrophoresis, leading to anomalous migration.	Substitute 7-deaza dGTP for dGTP in sequencing reactions to help disrupt secondary structure. (Table 5, pg. 82.)
		Increase the temperature of gel electrophoresis.
		Prepare the sequencing gel with 40% formamide.
Bands in all four lanes, occurring at specific regions.	Dissociation of enzyme from DNA template due to secondary structure in template.	Use *Taq* DNA polymerase. Because of its high sequencing temperature, *Taq* DNA polymerase can proceed through secondary structures that would cause Klenow, reverse transcriptase or T7 DNA polymerase to dissociate from the template. Perform a chase step to help eliminate false bands. After the termination reaction, cool the tubes to room temperature, add either dNTPs (2mM each) or an aliquot of the appropriate DNA polymerase (0.5-1.0 unit/tube) and incubate for 15 minutes at the appropriate sequencing temperature. Add stop solution after the chase step. Increase the incubation temperatures for Klenow (42-55°C) and reverse transcriptase (up to 50°C).

Technical Services:
800-356-9526

XII. Sequencing Primers: Sequences and Applications

The primers described in Table 13 are designed for sequencing inserts in a variety of vectors. Each pBR322 primer anneals to a region flanking one of four separate cloning sites: *Eco*R I/*Hind* III, *Pst* I, *Sal* I, or *Bam*H I. The SP6, T7 and T3 sequencing primers anneal to promoter sites flanking the multiple cloning regions of pGEM®, LambdaGEM® and pGEMEX™ vectors. The pUC/M13 primers are designed for sequencing inserts cloned into the M13 vectors and pUC plasmids developed by Messing (25), and can also be used for sequencing other *lacZ* containing plasmids such as the pGEM®-Z and pGEM®-Zf vectors. Lambda gt10 and gt11 primers are used for the direct sequence analysis of inserts cloned into the λgt10, λgt11, λgt11 *Sfi-Not*, and λgt22 cDNA cloning vectors.

Table 13. Sequencing Primer Specifications.

Primer	Orientation	Size	Sequence
pBR322 Primers			
*Eco*R I	cw	16mer	5′-d(GTATCACGAGGCCCTT)-3′
Hind III	ccw	16mer	5′-d(GCAATTTAACTGTGAT)-3′
Pst I	cw	16mer	5′-d(GCTAGAGTAAGTAGTT)-3′
Pst I	ccw	15mer	5′-d(AACGACGAGCGTGAC)-3′
Sal I	cw	15mer	5′-d(ATGCAGGAGTCGCAT)-3′
Sal I	ccw	15mer	5′-d(AGTCATGCCCCGCGC)-3′
*Bam*H I	cw	20mer	5′-d(CACTATCGACTACGCGATCA)-3′
*Bam*H I	ccw	16mer	5′-d(ATGCGTCCGGCGTAGA)-3′
SP6/T7 Primers			
SP6 Promoter	N/A	19mer	5′-d(GATTTAGGTGACACTATAG)-3′
T7 Promoter	N/A	20mer	5′-d(TAATACGACTCACTATAGGG)-3′
T3 Promoter	N/A	20mer	5′-d(ATTAACCCTCACTAAAGGGA)-3′
pUC/M13 Primers			
pUC/M13	Forward	17mer	5′-d(GTTTTCCCAGTCACGAC)-3′
pUC/M13	Reverse	17mer	5′-d(CAGGAAACAGCTATGAC)-3′
pUC/M13	Forward	24mer	5′-d(CGCCAGGGTTTTCCCAGTCACGAC)-3′
pUC/M13	Reverse	22mer	5′-d(TCACACAGGAAACAGCTATGAC)-3′
Lambda Primers			
Lambda gt11	Forward	24mer	5′-d(GGTGGCGACGACTCCTGGAGCCCG)-3′
Lambda gt11	Reverse	24mer	5′-d(TTGACACCAGACCAACTGGTAATG)-3′
Lambda gt10	Forward	27mer	5′-d(CTTTTGAGCAAGTTCAGCCTGGTTAAG)-3′
Lambda gt10	Reverse	31mer	5′-d(GAGGTGGCTTATGAGTATTTCTTCCAGGGTA)-3′

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XIV. Additional Nucleic Acid Sequencing Literature Available from Promega

Technical Bulletins

028	Sequencing of pGEM® dsDNA using AMV Reverse Transcriptase
030	GemSeq® Transcript Sequencing System
043	Sequencing Primers
050	α-Phosphorothioate Deoxynucleotides
058	Generation of ssDNA from pGEM®-Zf Phagemids

Manuals

TaqTrack® Sequencing System Technical Manual

T7 Sequencing System Technical Manual

Erase-a-Base® System Technical Manual

pGEM® Single Strand System Technical Manual

K/RT® System Technical Manual

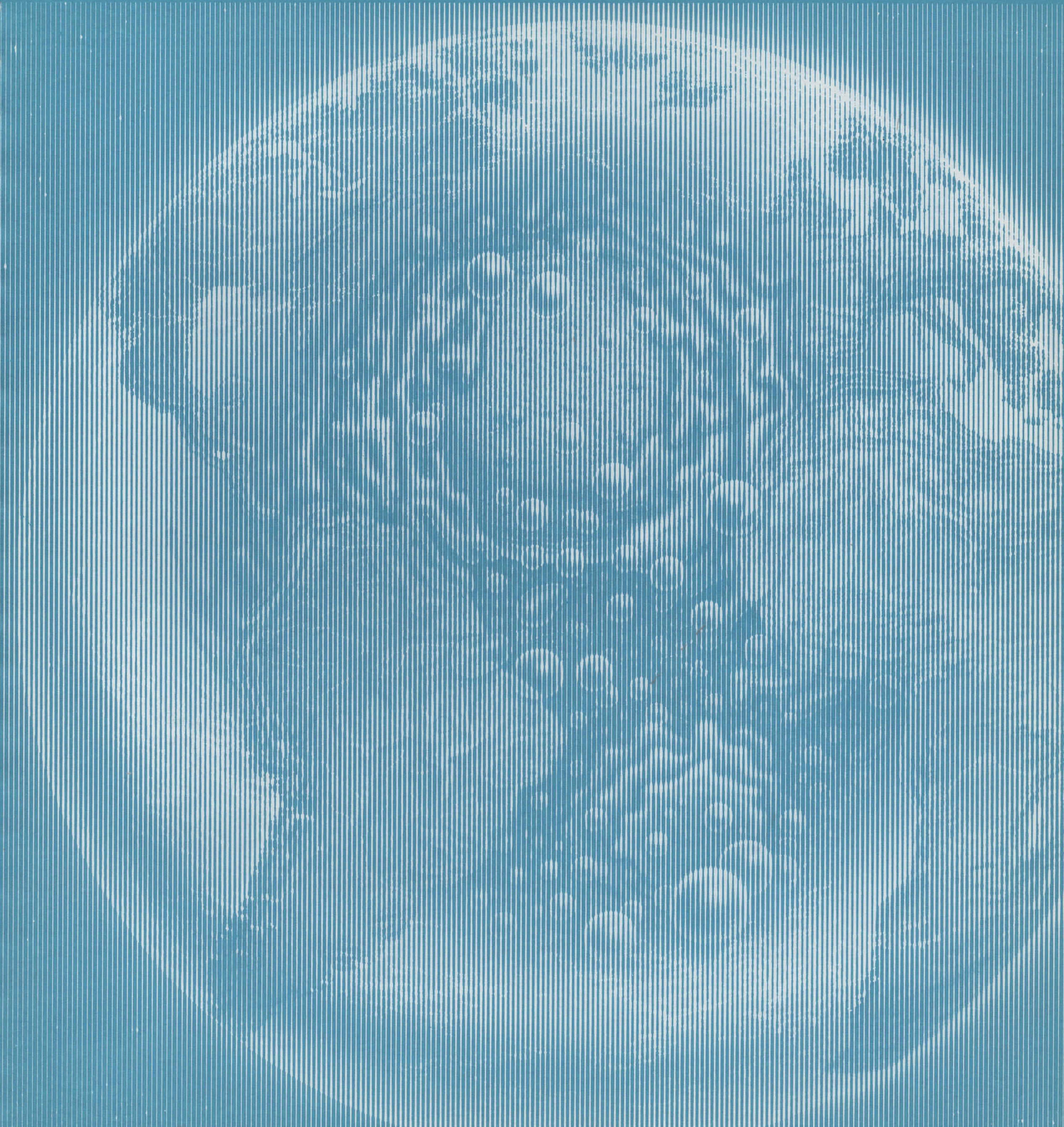
Altered Sites™ *in vitro* Mutagenesis System Technical Manual

Promega Notes Articles

Issue	Title
2	Direct sequencing of DNA cloned in Riboprobe® Gemini plasmids
3	GemSeq® Riboprobe Gemini sequencing system: technical update (mini-prep procedure)
4	GemSeq® Riboprobe Gemini sequencing system: technical update ^{35}S-ddNTP sequencing of supercoiled plasmid DNA
5	Direct sequencing of pGEM® plasmids: Klenow vs. RT
7	Sequencing of naturally occurring RNA using the GemSeq® transcription system-^{35}S-ddNTP sequencing of a plus-stranded RNA virus
8	Exonuclease III generated deletions for DNA sequence analysis
15	Phagemids: an alternative method for production of single-stranded DNA
16	TaqTrack® DNA sequencing system - introduction of a new, versatile catalyst for enzymatic sequence analysis
17	^{35}S sequencing using the TaqTrack® sequencing system
19	T7 DNA polymerase
20	T7 DNA polymerase sequencing systems
21	Erase-a-Base® System Update
22	Erase-a-Base® System: Maximizing Transformation Efficiency
22	AMV reverse transcriptase sequencing of ribosomal RNAs
23	A novel antibiotic selection method for site-directed mutagenesis: the Altered Sites™ *in vitro* mutagenesis system

Notes

PROTOCOLS AND APPLICATIONS GUIDE



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(continued)

I. Magnetic Particle Separation of Macromolecules

The attachment of nucleic acids to solid supports such as nitrocellulose (1,2) and cellulose (3) has found many applications in the field of molecular biology, particularly for affinity purification of proteins and nucleic acids. One common application of immobilized nucleic acids is oligo(dT) cellulose purification of messenger RNA (mRNA) by hybridization to the 3′ polyadenine tail (4). Recent years, however, have witnessed the emergence of paramagnetic particles as the solid support of choice for many affinity purification protocols. Paramagnetic particles incorporate iron oxide into submicron sized particles which have no magnetic field but form a magnetic dipole when exposed to a magnetic field. The use of paramagnetic particles eliminates the need for traditional column chromatography, centrifugation, or any other special equipment. These particles have been successfully used in the development of immunoassays (5), probe diagnostic assays (6), and for measuring RNA in cell lysates using dA-tailed capture probes (7).

Promega has extended the use of paramagnetic particles to the affinity purification of polyadenylated mRNA with the PolyATtract™ system and to cDNA synthesis and cloning with the Capture Clone™ system. Unlike procedures which use direct coupling of probes to paramagnetic particles (6,7), these systems use a biotinylated oligonucleotide probe to hybridize in solution to the targeted nucleic acid. The hybrids are then captured using covalently coupled streptavidin paramagnetic particles. This approach combines the speed and efficiency of solution hybridization with the convenience and speed (<1 minute) of magnetic separation.

Promega utilizes its own highly purified streptavidin for the production of the particles. These streptavidin paramagnetic particles (SA-PMPs) exhibit a high binding capacity for biotinylated oligonucleotides and low non-specific binding of nucleic acids. The binding capacity of the particles varies with the specific oligonucleotide probe used. For biotinylated oligo(dT), the calculated binding capacity is roughly 1nmole probe captured/mg SA-PMPs.

Promega

II. Rapid Isolation of Total RNA

A. Introduction

The purity and integrity of isolated RNA is critical for its effective use in procedures such as Northern blotting, oligo(dT) selection of poly(A)+ RNA, cDNA synthesis, and translation *in vitro*. This is especially true for the construction of cDNA libraries where efficient cDNA synthesis requires highly purified mRNA.

The successful isolation of intact RNA by any procedure requires that four important steps be performed: 1) effective disruption of cells or tissue, 2) denaturation of nucleoprotein complexes, 3) inactivation of endogenous ribonuclease (RNase) activity, and 4) purification of RNA away from contaminating DNA and protein. The most important of these is the immediate inactivation of endogenous RNase activity which is released from membrane bound organelles upon cell disruption. The RNAgents™ total RNA isolation kit utilizes two of the most potent known inhibitors of RNase, guanidine thiocyanate and β-mercaptoethanol (8). In addition, all procedures are done on ice, which significantly slows the rate of RNA degradation (9). Guanidine thiocyanate, in association with N-lauryl sarcosine, also acts to disrupt nucleoprotein complexes, allowing RNA to be released into solution and isolated free of protein.

Intact RNA is purified from contaminants by phenol/chloroform extraction (10) based on the rapid one-step procedure of Chomczynski and Sacchi (11) (Figure 1). RNA selectively partitions into the aqueous phase, free from DNA and protein, and is easily concentrated by precipitation with isopropanol. The procedure is easy to perform with large or small quantities of tissue or cultured cells and can be used to process multiple samples in as little as 3 hours. The use of phenol/chloroform extraction eliminates the need for lengthy selective ethanol precipitation steps, overnight ultracentrifugation through cesium chloride gradients, and the use of lithium chloride (LiCl) precipitations. LiCl precipitations can result in the loss of small RNAs (smaller than 5.8S) (12) and carryover of Li^+ salts can inhibit subsequent cDNA synthesis reactions (13). RNA purified by this technique can readily be used for purification of poly(A)+ RNA, Northern blots, cDNA synthesis, and translation *in vitro*.

B. Creating a Ribonuclease-Free Environment

Ribonuclease is difficult to inactivate. Therefore it is of utmost importance that care be taken to avoid inadvertently introducing RNase activity into your RNA during or after the isolation procedure. This is especially important if the starting material has been difficult to obtain and may not be able to be replaced. The following notes will help you to prevent accidental contamination of your sample.

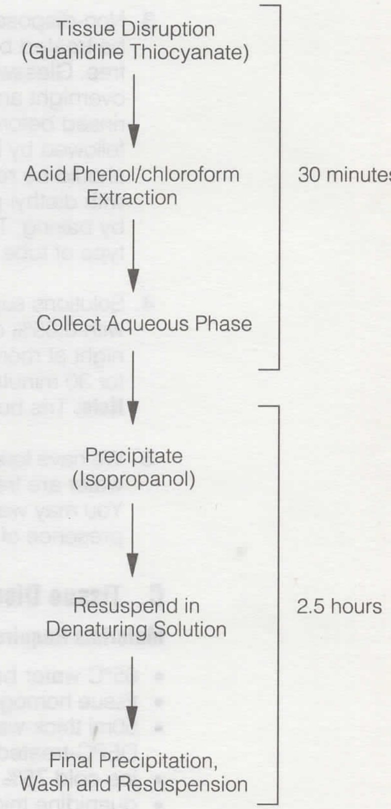

Figure 1. Outline of the RNAgents total RNA isolation procedure.

II. Rapid Isolation of Total RNA

(continued)

1. Two of the most common sources of RNase contamination are the user's hands and bacteria and molds that may be present on airborne dust particles. To prevent this type of contamination, proper microbiological sterile technique should be observed when handling the reagents supplied with the kit and gloves should be worn at all times.

2. Whenever possible, sterile disposable plasticware should be used for handling RNA. These materials are generally RNase-free and thus do not require pretreatment to inactivate RNase.

3. Non-disposable glass- and plasticware should be treated before use to ensure that it is RNase-free. Glassware should be baked at 200°C overnight and plasticware should be thoroughly rinsed before use with 0.1N NaOH, 1mM EDTA followed by RNase-free water. **Note:** Corex tubes should be rendered RNase-free by treatment with diethyl pyrocarbonate (see below) and not by baking. This will reduce the failure rate of this type of tube during centrifugation.

4. Solutions supplied by the user should be treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature and then autoclaved for 30 minutes to remove any trace DEPC. **Note:** Tris buffers can not be treated with DEPC.

5. We have found that many good sources of distilled water are free of contaminating RNase activity. You may want to test your water source for the presence of RNase activity.

C. Tissue Disruption

Materials Required

- 65°C water bath or oven
- tissue homogenizer (such as Polytron or Dounce)
- 50ml thick-walled polypropylene tubes, DEPC-treated
- ice-cold 75% ethanol
- guanidine thiocyanate (supplied)
- CSB buffer (supplied, pg. 130)
- 2M sodium acetate, pH 4.0 (supplied)
- phenol:chloroform:isoamyl alcohol (25:24:1) (supplied)
- isopropanol (supplied)
- RNase-free water (supplied)

For cultured cells:

- PBS 10X buffer (pg. 130)

Storage: All of the kit components may be stored at room temperature. However, storage of phenol:-chloroform:isoamyl alcohol at 4°C is recommended for increased stability.

Preparations

- Pretreat the 50ml thick-walled polypropylene centrifuge tubes in 0.05% diethyl pyrocarbonate (DEPC) for 1 hour at room temperature and then autoclave the tubes for 30 minutes to destroy any residual DEPC.
- Allow the phenol:chloroform:isoamyl alcohol solution to stand at room temperature for 15 minutes to allow the phases to separate. The phases may have mixed during shipping.
- Prepare the denaturing solution by adding 33ml of CSB buffer (pg. 130) to one of the 25g bottles of guanidine thiocyanate. Mix thoroughly until all of the components are completely dissolved. The solution may be heated in a 65°C water bath if one is available. Store at 4°C for up to three months. **Note:** N-lauryl sarcosine may precipitate out of solution. If this occurs, heat to redissolve before using.

Caution: Guanidine thiocyanate is a potent chaotropic agent and irritant. In addition, phenol is poisonous and can cause severe burns. Proper laboratory clothing including gloves and goggles should be worn when handling these reagents. If phenol should contact your skin, rinse the area immediately with large quantities of water and seek medical attention. **DO NOT RINSE WITH ETHANOL!** For more complete precautions, refer to the appropriate material safety data sheet (MSDS) supplied with the kit.

Tissue Disruption

The samples to be extracted should be as fresh as possible to obtain optimum RNA yields. However, if it is not possible to isolate RNA immediately after obtaining the sample of interest, the sample may be frozen with liquid nitrogen and stored at –70°C to be used at a later date. The procedures described below are intended for isolating RNA from 1 gram of tissue or 10^8 cultured cells. The volumes of reagents may be adjusted proportionally for different amounts of starting material.

II. Rapid Isolation of Total RNA

(continued)

1. Cells Grown in Suspension Culture

Collect 1 x 10^8 cells in a sterile 50ml conical centrifuge tube by centrifugation at 300 x g for 5 minutes at 4°C. Wash the cell pellet with 25ml of ice cold, sterile 1X PBS (pg. 130) and centrifuge as above to collect the cells. Pour off the supernatant, add 15ml of prechilled denaturing solution (pg. 130), and homogenize as described for animal tissue, Step 3.b. Next, proceed to Step D.1, below.

2. Cultured Cells Grown in a Monolayer

Different cell lines will grow to different cell densities. Calculate the total number of flasks needed to provide approximately 1 x 10^8 cells. Pour off the culture media from each flask and wash the cells with ice cold, sterile 1X PBS (pg. 130).

a. Add 8ml of prechilled denaturing solution (pg. 130) to one of the flasks and rotate it over the cells until you see them lyse. The solution will become very viscous.

b. Transfer the solution from flask #1 to flask #2 using a sterile 10ml glass pipet, lyse the cells, transfer the solution to flask #3, lyse the cells, and so on depending on the total number of flasks.

c. Add 4ml of denaturing solution to flask #1. Rotate the solution thoroughly over the bottom of the flask to remove any remaining cells and then transfer this solution successively to the other flasks. **Note:** If you feel that all of the cells have not been removed, use an additional 5-10ml of denaturing solution to wash the flasks. In this case, however, the amounts of other reagents to be added subsequently will have to be increased proportionately.

d. Transfer the 12ml of lysed cells from the last flask to a sterile 50ml conical centrifuge tube and homogenize the cells as described in Step 3.b. Proceed to Step D.1, below.

3. Animal Tissue

a. Dispense 12ml of denaturing solution (pg. 130) into a sterile 50ml cell culture tube and chill on ice for 5 minutes.

b. Place 1 gram of tissue (either fresh or frozen) into the denaturing solution and disrupt the tissue with a high speed homogenizer such as the Brinkman Polytron set on high for 15-30 seconds. This usually can be performed in a sterile 50ml conical cell culture tube. Alternatively, the tissue can be minced and disrupted with a Dounce glass-teflon homogenizer. Proceed to step D.1, below.

D. RNA Extraction and Precipitation

1. Add 1.2ml of 2M sodium acetate, pH 4.0 and mix thoroughly by inversion.

2. Add 12ml of phenol:chloroform:isoamyl alcohol mixture, mix by inversion and shake vigorously for 10 seconds. Chill on ice for 15 minutes.

3. Transfer this mixture to a 50ml thick-walled polypropylene tube (DEPC-treated) and centrifuge at 10,000 x g for 20 minutes at 4°C. Either a fixed-angle or swinging bucket rotor may be used.

4. Carefully remove the *TOP AQUEOUS PHASE* which will contain the RNA and transfer it to a fresh DEPC-treated tube. DNA and proteins will remain in the organic phase and at the interface. Be careful to avoid taking material from the interface.

Nucleic Acid Detection, Purification and Labeling

II. Rapid Isolation of Total RNA

(continued)

RNA Precipitation

5. Add an equal volume of isopropanol and incubate the sample at –20°C for at least 30 minutes to precipitate the RNA. **Note:** To obtain the maximum RNA yield from tissue samples that contain relatively low amounts of RNA, we recommend longer precipitations (up to overnight).

6. Pellet the RNA by centrifugation at 10,000 x g for 15 minutes at 4°C.

7. Resuspend the RNA pellet in 5ml of denaturing solution and vortex until the RNA is dissolved. In some instances, heating to 65°C may be required to resuspend the pellet. Heating should be done for as short a time as possible.

8. Add an equal volume of isopropanol and precipitate the RNA as described in Step 5, above.

RNA Wash

9. Pellet the RNA by centrifugation at 10,000 x g for 15 minutes at 4°C. Wash the pellet with ice-cold 75% ethanol and centrifuge as above. A minimum of 10ml of 75% ethanol should be used.

10. Dry the pellet in a vacuum desiccator for 15-20 minutes. Do not let the pellet completely dry out as this will make the pellet very difficult to resuspend.

11. Resuspend the RNA in 1-3ml of RNAse-free deionized H_2O and store at –20°C. For long term storage, we recommend that the RNA be reprecipitated with ethanol; add sodium acetate, pH 5.0 to 0.25M and then add 2.5 volumes of ethanol. Storage should be at –70°C.

Procedures for isolation of poly(A)+ mRNA are provided in Sections III (pg. 130) and IV (pg. 134).

E. Determination of RNA Quality

Purity

RNA properly isolated with the RNAgents kit is substantially free of DNA and contaminating protein and may be used directly for oligo(dT) selection of poly(A)+ RNA, Northern blots, cDNA synthesis, and translation *in vitro*. Pure RNA will exhibit A_{260}/A_{280} ratios of 2.0. However it should be noted that, due to the variations between different starting materials and individual variation in performing the procedure, you should expect to obtain RNA having A_{260}/A_{280} ratios ranging from 1.7-2.0. If the RNA exhibits a ratio lower than this, refer to the troubleshooting section, below, for ways to further purify the RNA.

The isolated RNA should also exhibit an A_{260}/A_{230} ratio greater than 2.0. A ratio lower than this is generally indicative of contamination with guanidine thiocyanate that was carried over during the precipitation steps (see Troubleshooting, below).

Integrity

Determine the integrity of the purified RNA by denaturing agarose gel electrophoresis. Several methods are suitable for this purpose, utilizing either formaldehyde (14), glyoxal (15), or methylmercury hydroxide (16). When resolved by one of these electrophoretic methods, the 28S and 18S eukaryotic ribosomal RNAs should exhibit a near 2:1 ratio of ethidium bromide staining, indicating that no gross degradation of RNA has occurred. In RNA samples that have been degraded, this ratio will be reversed since the 28S ribosomal RNA is characteristically degraded to an 18S-like species. Refer to Section B (pg. 125), and the troubleshooting section, below, for ways to avoid RNA degradation.

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II. Rapid Isolation of Total RNA

(continued)

F. Troubleshooting

Problem	Recommendation
Low A_{260}/A_{280} ratios.	Typically due to protein contamination. Several methods may be used for further removal of contaminating protein from RNA. The most expedient method is to perform an additional phenol/chloroform extraction on the purified RNA. Repeat Steps D.1-D.6 (pg. 127), adding a volume of phenol/chloroform equal to that of the final resuspended RNA pellet. This procedure should yield higher A_{260}/A_{280} ratios. However, some loss of RNA (up to 40%) may be expected.
Low A_{260}/A_{230} ratios.	Typically due to guanidine thiocyanate contamination. Precipitate the RNA by adding 0.1 volume of 2M sodium acetate, pH 4.0 and an equal volume of isopropanol. Incubate at –20°C for 30 minutes and collect the RNA by centrifugation at 10,000 x g for 15 minutes at 4°C. Resuspend the RNA in 1mM EDTA (RNase-free) and precipitate the RNA as described above. Wash the final pellet with 10ml of 75% ethanol, dry the pellet in a vacuum desiccator for 15-20 minutes, and resuspend in RNase-free deionized H_2O.
RNA degradation.	RNase introduced by handling or not all RNase inactivated. One of the common misconceptions about RNA isolation is that RNase is irreversibly denatured with guanidine thiocyanate salts. If a small amount of denatured RNase is present at the end of the procedure, it may renature once the denaturing agents are removed and degrade the sample. This is most likely to occur with samples that contain very high amounts of endogenous RNase, such as rat pancreas. If this is a problem, use the following procedure, which is based on the isolation protocol of Han *et al.* (9) to eliminate such carryover RNase activity in subsequent RNA isolations. a. After the RNA has been precipitated for the second time from denaturing solution (Step D.10, pg. 128), resuspend the pellet in 10ml of prechilled (4°C) 6M guanidine-HCl solution. This can be prepared by dissolving 57g of guanidine-HCl in 25ml of 0.1M EDTA, pH 7.0, adding 75µl of β-mercaptoethanol, and then adding H_2O to a final volume of 100ml. The solution should be filtered before use (0.45µm pore size). Vortex the RNA sample vigorously to resuspend the pellet. If not all of the pellet will go into solution, the pellet may be physically broken up with an RNase-free sterile glass rod. It is important to solubilize as much of the pellet as possible to maximize the recovery of your RNA. b. Precipitate the RNA by adding 0.05 volume of 1M acetic acid and 0.75 volume of 100% ethanol. Incubate at –20°C for at least 15 minutes. c. Collect the RNA by centrifugation at 10,000 x g for 10 minutes at 4°C. Pour off the supernatant. d. Repeat Steps a-c two more times, reducing the volume by one half with each round of resuspension and precipitation. e. Wash the final pellet with ice-cold 75% ethanol, dry the pellet in a vacuum desiccator for 15-20 minutes, and resuspend in RNase-free deionized H_2O.

Nucleic Acid Detection, Purification and Labeling

II. Rapid Isolation of Total RNA

(continued)

Composition of Solutions

PBS 10X buffer (per liter):

11.5g	Na_2HPO_4
2g	KH_2PO_4
80g	NaCl
2g	KCl

Add sterile, deionized water to 1 liter final volume. The pH of 1X PBS will be 7.4.

Denaturing solution:

25g	guanidine thiocyanate (4M final)
33ml	CSB buffer

Mix thoroughly until components are completely dissolved. The solution may be heated in a 65°C water bath to help dissolution. Store at 4°C.

CSB buffer:

42mM	sodium citrate
0.83%	N-lauryl sarcosine
0.2mM	β-mercaptoethanol

III. Magnetic Isolation of Poly(A)+ RNA

A. Introduction

The PolyATtract™ mRNA isolation system utilizes paramagnetic particle technology to eliminate the need for oligo(dT) cellulose and its associated problems. With total RNA as the starting material, the poly(A)+ mRNA fraction can be isolated free of all other nucleic acid contamination in approximately 45 minutes. By contrast, a standard oligo(dT) cellulose selection procedure (pg. 134) requires 1.5 hours to prepare the resin and an additional 1.5 hours to perform the mRNA isolation. When used in a column, oligo(dT) cellulose is also prone to clogging. Magnetically isolated mRNA is suitable for all molecular biology applications, including translation *in vitro* and cDNA synthesis.

The system uses a biotinylated oligo(dT) primer to hybridize at high efficiency in solution to the 3′ poly(A) region present in most mature eukaryotic mRNA species. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic separation stand included with the system (Figure 2). The mRNA is eluted from the solid phase by the simple addition of ribonuclease-free deionized water. This procedure yields an essentially pure fraction of mature mRNA after only a single round of purification (Figures 2 and 3).

All of the components in the kit are guaranteed to be free of contaminating ribonucleases and have been thoroughly tested to ensure optimal performance. When used in combination with Promega's RNAgents™ total RNA isolation kit, a pure fraction of intact, full length mRNA can be isolated from a tissue or cell source in as little as 4 hours.

B. Magnetic mRNA Isolation Procedure

Total RNA to be used in this procedure should be dissolved in deionized, RNase-free water or 0.5% SDS and taken up in deionized water to a final

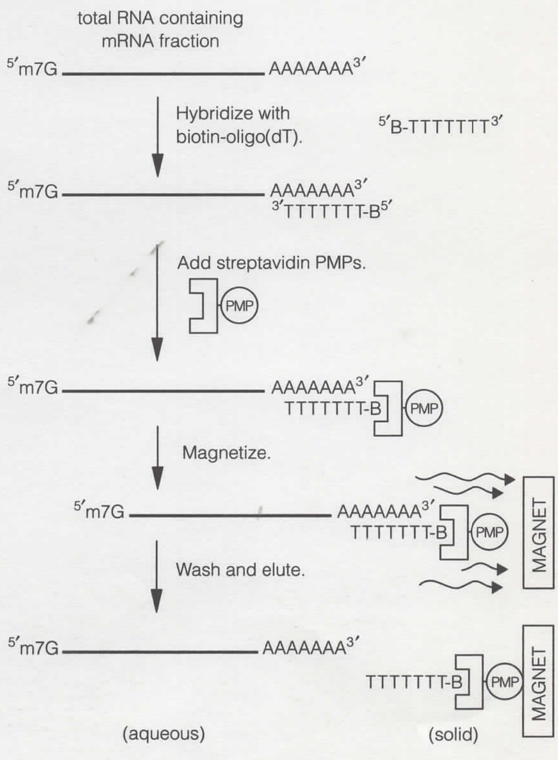

Figure 2. Schematic diagram of the PolyATtract mRNA isolation system.

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III. Magnetic Isolation of Poly(A)+ RNA

(continued)

volume of 2.43ml (Step A.1, below). To avoid RNase contamination, review the precautions listed in Section II.B., pg. 125.

Materials Required

- 65°C water bath or heating block
- sterile, RNase-free, 5ml and 15ml glass or plastic tubes
- sterile, RNase-free pipets and pipet tips
- biotinylated oligo(dT) probe, 50pmole/µl (supplied)
- 20X SSC solution (pg. 134, supplied)
- streptavidin MagneSphere™ particles (supplied)
- RNase-free water (supplied)

Storage and Stability: The components supplied are stable for six months from the date of purchase if stored at 4°C. **Do not freeze or heat the Streptavidin MagneSphere™ particles (SA-PMPs), as this will reduce their performance.** The other components may be frozen.

Annealing of Probe

1. In a sterile, RNase-free 5ml tube, combine up to 5mg of total RNA and RNase-free water to a final volume of 2.43ml.

2. Place the tube in a 65°C water bath for 10 minutes.

3. Add 10µl of the biotinylated-oligo(dT) probe and 60µl of 20X SSC (pg. 134) to the RNA. Mix gently and incubate at room temperature until completely cooled. This may require up to 30 minutes, depending on the size of the tube. While this solution is cooling, prepare stock solutions of 0.5X and 0.1X SSC (Steps 4 and 5).

Stock Solution Preparation

4. Prepare 5ml of sterile 0.5X SSC by combining 0.125ml of 20X SSC with 4.875ml of RNase-free water in a sterile, RNase-free tube.

5. Prepare 10ml of sterile 0.1X SSC by combining 50µl of 20X SSC with 9.55ml of RNase-free water in a sterile, RNase-free tube.

Washing of Streptavidin-Paramagnetic Particles

6. Resuspend the SA-PMPs by gently flicking the bottom of the tube until they are completely dispersed and then capture them by placing the tube in the magnetic rack until the SA-PMPs have collected at the side of the tube (approximately 30 seconds). Carefully remove the supernatant. Do not centrifuge the particles.

7. Wash the SA-PMPs three times with 0.5X SSC (1.5ml per wash), each time capturing them using the magnetic rack and then carefully removing the supernatant.

8. Resuspend the washed SA-PMPs in 0.5ml of 0.5X SSC.

Note: The SA-PMPs should be used soon after washing, as they are less stable in the absence of protein.

Capture and Washing of Annealed Oligo(dT)-mRNA Hybrids

9. Add the entire contents of the annealing reaction to the tube containing the washed SA-PMPs.

10. Incubate at room temperature for 10 minutes.

11. Capture the SA-PMPs using the magnetic rack and carefully remove the supernatant without disturbing the SA-PMP pellet. **Note:** Good laboratory practice dictates that this supernatant be saved until you are certain that satisfactory binding and elution of mRNA has occurred.

12. Wash the particles four times with 0.1X SSC (1.5ml per wash) by gently flicking the bottom of the tube until all of the particles are resuspended. After the final wash, remove as much of the aqueous phase as possible without disturbing the particles.

Elution of mRNA

13. To elute the mRNA, resuspend the final SA-PMP pellet in 1ml of the RNase-free water supplied with the system and gently resuspend the particles by flicking the tube.

14. Magnetically capture the SA-PMPs and remove the eluted mRNA aqueous phase into one of the 2ml tubes provided. Do not throw the particles away.

Note: If any of the particles are carried over at this point they may be removed by centrifuging at 10,000 x g for 5-10 minutes at 4°C. Carefully remove the RNA and place it into a fresh RNase-free tube.

Nucleic Acid Detection, Purification and Labeling

III. Magnetic Isolation of Poly(A)+ RNA

(continued)

C. Determination of mRNA Concentration and Purity

The concentration and purity of the eluted mRNA can be determined by spectrophotometry. Pure mRNA will have an A_{260}/A_{280} absorbance ratio of ≥2.0. To estimate the mRNA concentration, assume that a 40µg/ml mRNA solution will have an absorbance of 1 at 260nm. The quality of the isolated mRNA may also be checked by denaturing agarose gel electrophoresis (17). Figure 3 illustrates the appearance of the mRNA fraction isolated from total mouse liver RNA using the PolyATtract system.

The mRNA should appear as a smear extending from approximately 8.0kb to approximately 0.5kb (depending on the tissue). The bulk of the mRNAs should be clustered around 2.0kb. We normally see very little ribosomal RNA contamination after the first round of selection. However, the appearance of some ribosomal bands does not indicate poor performance of the system. Figure 4 shows a Northern blot of mRNA which contains visible amounts of both 28S and 18S ribosomal RNAs. The blot was probed with ^{32}P-labeled biotinylated oligo(dT). Little or no hybridization is seen in the total RNA lane, whereas the mRNA selected material shows a tremendous enrichment despite the presence of some ribosomal RNAs. Therefore, a small amount of ribosomal contamination should not affect the functionality of the mRNA and it is suitable for most applications. If your application requires extensive purification, excess ribosomal RNA may be removed by an additional selection, described below.

Optional Additional Purification

1. Heat the 1ml of eluted mRNA to 65°C for 10 minutes then add an additional 10µl of probe plus 20X SSC to a final concentration of 0.5X. Continue the isolation from Step B.6 using a fresh tube of particles.

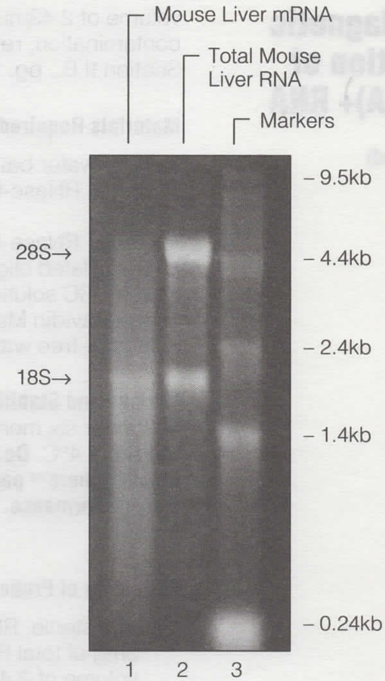

Figure 3. Isolation of mRNA using the PolyATtract mRNA isolation system. The PolyATtract system was used to isolate highly enriched, undegraded mRNA from total mouse liver RNA which had been prepared using the RNAgents kit. RNA samples (5µg per lane) were resolved on a 1% denaturing agarose gel. Lane 1, mRNA fraction; lane 2, total mouse liver RNA; lane 3, 0.24-9.5kb RNA molecular weight standards. Arrows indicate the positions of the 28S and 18S ribosomal bands.

III. Magnetic Isolation of Poly(A)+ RNA

(continued)

D. Precipitation and Concentration of mRNA

While the mRNA fraction isolated with the PolyATtract™ system is sufficiently concentrated for spectrophotometric analysis, it may be too dilute for applications such as cDNA cloning and translation *in vitro*. The RNA may be concentrated by alcohol precipitation (13).

1. **For cDNA cloning:** Add 0.1 volume of 3M sodium acetate and 1.0 volume of isopropanol to the eluate and incubate at –20°C overnight.

   For translation *in vitro*: Add 0.1 volume of 3M potassium or ammonium acetate and 1.0 volume of isopropanol to the eluate and incubate at –20°C overnight.

2. Centrifuge at >12,000 x g for 10 minutes. Resuspend the RNA pellet in 1ml of 75% ethanol and centrifuge again.

3. **For short-term storage:** Dry the pellet in a vacuum desiccator for about 15 minutes, resuspend in RNase-free, deionized water at 0.5-1.0µg/µl, and store at –70°C.

   For long-term storage: Store the RNA pellet in 75% ethanol at –70°C.

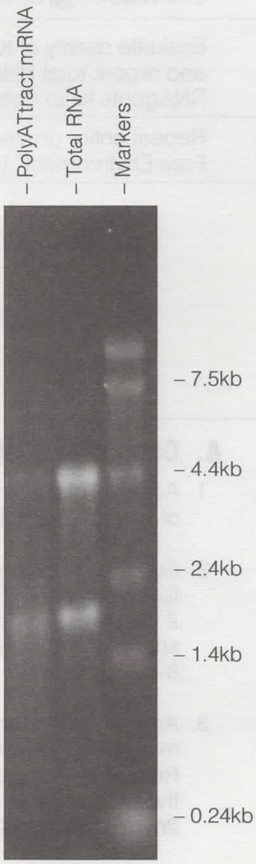

Figure 4A

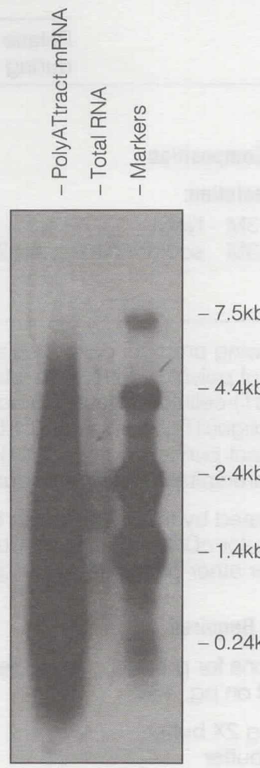

Figure 4B

Figure 4, A and B. Analysis of purified mRNA using the PolyATtract mRNA isolation system. (A) Ethidium bromide stained RNA samples (5µg per lane) on a 1% denaturing agarose gel. Lane 1, mRNA fraction; lane 2, total mouse liver RNA; lane 3, 0.24-9.5kb RNA molecular weight standards. (B) RNAs shown in panel A were blotted to nitrocellulose and probed with ^{32}P-labeled biotinylated oligo(dT) at 50°C in 6X SSC containing 1X Denhardt's solution and 0.1% SDS. The results of the autoradiogram are shown.

III. Magnetic Isolation of Poly(A)+ RNA

(continued)

E. Troubleshooting

Problem	Possible Causes	Comments
No mRNA eluted.	No mRNA bound due to salt omitted from annealing procedure.	Repeat annealing step, adding 20X SSC (to 0.5X final).
	Insufficient cooling of annealing reaction before probe capture and wash.	Add the saved supernatant back to the particles as in Step B.9 (pg. 131) and continue with the procedure.
	Salt not eliminated during elution.	Wash last SA-PMP pellet again with deionized H_2O and check A_{280} of this eluate.
RNA appears degraded on gel.	RNase contamination in total RNA.	Evaluate quality of total RNA by gel electrophoresis and repeat total RNA isolation as necessary. Use RNAgents kit to obtain high quality total RNA.
	RNase contamination during mRNA isolation.	Repeat entire procedure. Read "Creating a RNase-Free Environment" (pg. 125).

Solution Composition

20X SSC solution:

3M	NaCl
0.3M	sodium citrate, pH 7.0

IV. Oligo(dT) Cellulose Isolation of Poly(A)+ RNA

The following protocol describes a method for isolation of poly(A)+ RNA from total RNA by binding to oligo(dT)-cellulose. Allow 1.5 hours for the preparation of oligo(dT) cellulose and 1.5 hours for the subsequent purification of poly(A)+ RNA. A final ethanol precipitation step is performed overnight.

RNA isolated by this method can be used as a substrate for cDNA library construction, translation *in vitro*, or other biological techniques.

Reagents Required

Instructions for preparing these reagents are provided on pg. 135.

- binding 2X buffer
- wash buffer
- elution buffer
- TE buffer
- sodium acetate (2M and 3M)
- 0.1N NaOH
- ethanol (100% and 70%)

A. Oligo(dT) Cellulose Preparation

1. Add 2ml of binding 1X buffer (pg. 135) to 0.6g of oligo(dT)-cellulose type 7.
2. Add 2ml of 0.1N NaOH to the resin and mix gently for a few minutes. Centrifuge at 1,500 x g for 2 minutes (see pg. 135, Note 2). Remove supernatant and discard. Repeat procedure 8-10 times.
3. Add 2ml of binding 1X buffer to the resin and mix gently. Centrifuge at 1,500 x g for 2 minutes. Remove the supernatant and discard. Repeat the procedure 8-10 times. Resuspend gently in 2ml of binding 1X buffer.
4. Transfer 1ml of the slurry to each of 2 microcentrifuge tubes. One of these tubes may be stored, for future use, at 4°C with the addition of 0.5ml of binding 1X buffer and 0.05% sodium azide.

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IV. Oligo(dT) Cellulose Isolation of Poly(A)+ RNA

(continued)

B. mRNA Isolation Procedure

1. Add 1 volume of binding 2X buffer to 100-300µl of the total RNA prepared in Step D.11 (pg. 128). Heat the mixture for 10 minutes at 65°C and then allow to cool to room temperature.

2. Add the RNA mixture to the 1ml slurry of oligo(dT) in binding 1X buffer.

3. Shake gently for 15 minutes at room temperature to allow the RNA to bind to the resin.

4. Centrifuge at 1,500 x g for 5 minutes and discard the supernatant.

5. Add 500µl of wash buffer (below) to the resin. Shake gently for a few seconds. Centrifuge at 1,500 x g for 2 minutes. Discard the supernatant. Repeat this procedure 3 more times.

6. Add 200µl of elution buffer (below) to the resin and shake gently for a few minutes. Centrifuge at 1,500 x g for 5 minutes. Transfer the supernatant to a fresh tube. Repeat this procedure 2 more times, saving the supernatants each time.

7. Add 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol to the pooled supernatants. Mix and leave at –20°C overnight.

8. Centrifuge at 12,000 x g for 15 minutes. Wash the RNA pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and resuspend in an appropriate volume of TE buffer. Store at –70°C.

Notes:

1. Centrifugation greater than 1,500 x g may result in damage to the oligo(dT) beads.

2. Oligo(dT) will bind poly(A)+ mRNA at a ratio of 0.5mg RNA/0.3g resin.

Composition of Solutions

Binding 2X buffer:

0.02M	Tris-HCl, pH 7.5
1M	NaCl
2mM	EDTA
1.0%	SDS

Wash buffer:

0.01M	Tris-HCl, pH 7.5
0.1M	NaCl
1mM	EDTA

Elution buffer:

0.01M	Tris-HCl, pH 7.5
1mM	EDTA

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

*Buffer tablets available from Promega.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

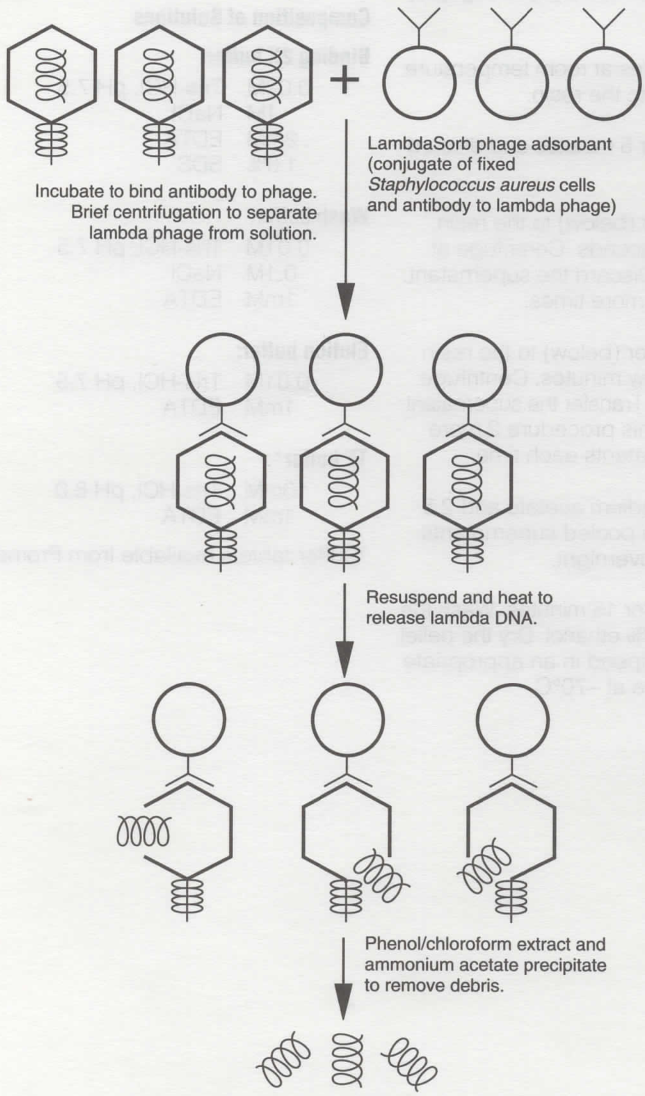

Figure 5. Schematic of lambda DNA purification using LambdaSorb phage adsorbent.

Lambda phage lysates suitable for DNA isolation can be made from either plate or liquid cultures. Protocols for preparing plate lysates or liquid culture lysates are provided in Sections A and B, below. While both methods work well, it is easier to monitor the growth of phage with the plate method while the liquid culture method is somewhat easier to scale up. Allow 7-9 hours for the plate method and 55-60 hours for the liquid culture method.

Recombinant lambda DNA may be purified from phage lysates using LambdaSorb® phage adsorbent (Section C, pg. 138) or by using a mini-prep protocol (Section D., pg. 140).

LambdaSorb phage adsorbent is designed for the rapid purification of bacteriophage lambda DNA from crude mini-preps. The reagent consists of an optimized conjugate of fixed *Staphylococcus aureus* cells and rabbit polyclonal antibodies directed against lambda bacteriophage particles. In practice, the adsorbent is simply added to crude lysates, incubated for 30 minutes, and phage are collected by centrifugation (Figure 5). The high degree of specificity of this initial step eliminates the need for extensive subsequent purification steps usually included in lambda mini-prep protocols. The DNA is freed from the precipitated complex by heating in the presence of EDTA and then is extracted with phenol/chloroform and ethanol precipitated. The result is a high yield of clean lambda DNA which is amenable to restriction enzyme analysis and sequencing.

A. Plate Method for Phage Lysate Preparation

Reagents Needed

- SM buffer (pg. 140)
- LB plates and top agarose (pg. 141)
- chloroform

Protocol

1. For each 100mm plate, mix about 1 x 10^5 pfu of bacteriophage (or 60-100µl of a normal phage "plug" left to elute 1 hour at room temperature in 1ml of SM buffer) with 0.1ml of a fresh overnight culture of appropriate plating bacteria. Incubate at 37°C for 20 minutes.

2. Add 2.5ml of melted top agarose (0.6% agarose in LB medium, pg. 141) at 47°C, mix, and pour onto a plate containing fresh, hardened LB bottom agar.

   Note: Top *agar* is not used due to the presence of inhibitors which interfere with restriction enzyme digestions.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

(continued)

3. Invert the plates and incubate at 37°C for 6 hours or until the plaques become confluent.

4. Overlay each plate with 2-3ml of SM buffer (pg. 140). Scrape the top agarose with a spatula into a high speed 30-50ml centrifuge tube, breaking up the agarose with the spatula.

5. Incubate at room temperature with intermittent shaking for 30 minutes.

6. Centrifuge at 8,000-10,000 x g for 10 minutes at 4°C.

7. Carefully withdraw the supernatant and transfer to a fresh tube. Add chloroform to 0.3% if long term storage is desired. Proceed to Section C or D for isolation of lambda DNA.

B. Liquid Culture Method for Phage Lysate Preparation

Reagents Needed

- phage buffer (pg. 141)
- LB medium (pg. 141)
- 1M $MgSO_4$
- 20% (w/v) maltose
- chloroform

Protocol

1. Pick a single phage plaque from an agar plate by removing a plug of agar containing the plaque with a wide bore borosilicate Pasteur pipet or a disposable micropipet tip with the end cut off. Gently expel the agar plug into a 1.5ml microfuge tube containing 100µl of phage buffer (pg. 141). Place at 4°C overnight. Since lambda phage can diffuse rapidly through the top agar (17), it is best to choose well isolated plaques from a fresh plate.

2. Start a fresh culture of an appropriate host strain (e.g., LE392) by inoculating a single colony into 5ml of LB medium supplemented with 50µl of 1M $MgSO_4$ and 50µl of 20% (w/v) maltose. Shake (2,400rpm) overnight at 37°C.

3. Add 500µl of the overnight culture to the tube containing the top agar plug. Incubate for 20 minutes at 37°C.

4. Use the above to inoculate 100ml of prewarmed (37°C) LB medium (supplemented with 1ml of 1M $MgSO_4$) in a 250ml Erlenmeyer flask. Shake (2,400rpm) at 37°C until lysis occurs (about 3.5 hours). The medium should appear cloudy after two hours of incubation and should clear upon cell lysis. Cellular debris should be visible in the lysed culture. If lysis has not occurred after 7 hours, add 500µl of chloroform and continue to shake for 15 minutes.

5. Spin down cellular debris at 8,000 x g for 10 minutes. Transfer the supernatant to a sterile tube. Store at 4°C.

6. The phage DNA may be purified from the lysate medium using LambdaSorb® phage adsorbent (Section C, below) or using a mini-prep protocol (Section D, pg. 140).

Note: Lysate phage titers are typically on the order of 10^9-10^{10} plaque forming units (pfu) per ml. Additional information on the preparation of lambda lysates may be found in reference 18.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

(continued)

C. DNA Isolation using LambdaSorb® Phage Adsorbent

Using LambdaSorb phage adsorbent (Figure 5, pg. 136), 100ml of lysate (at a titer of 5×10^9 pfu/ml) should yield approximately 12-20μg DNA. **Note:** When starting with less than 20ml lysate, it may be more convenient to carry out Steps 4-12 in microcentrifuge tubes. In this case, the centrifugation steps should be performed for 5 minutes in a microcentrifuge (>12,000 x g). Phage DNA isolated by the plate method is often difficult to digest with *Eco*R I or other restriction enzymes unless a PEG precipitation step is included in this protocol. For this reason, two alternative DNA precipitation methods are given in Step 11.

Reagents Needed

Instructions for preparing these reagents are provided on pg. 140.

- SM buffer
- release buffer
- 5M NaCl
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)
- TE buffer
- 30% PEG-8000 in 1.5ml NaCl
- DNase-free RNase A

Protocol

1. Resuspend by inversion the LambdaSorb phage adsorbent which has settled out during storage.
2. Add 100μl of adsorbent per 10ml of phage lysate in a 15 or 50ml high speed centrifuge tube. Mix for 30 minutes on a shaker at room temperature.
3. Centrifuge at 10,000 x g for 15 minutes. Carefully pour off the supernatant. (To test the efficiency of the adsorption step, save the supernatant at 4°C and check the titer at a later time.)
4. Resuspend the pellet in 1ml of SM buffer per 10ml initial phage lysate (Step C.1, above) by pipetting up and down.
5. Spin the suspension at 10,000 x g for 10 minutes. Aspirate the supernatant.
6. Repeat Steps 4 and 5.
7. Resuspend the pellet in 0.5ml of release buffer per 10ml of initial phage lysate (Step C.1). Heat at 67°C for 5 minutes to release the phage DNA.
8. Spin for 10 minutes at 10,000 x g to remove the adsorbent. Transfer the supernatant to another tube.
9. Add 5μl of 5M NaCl per 0.5ml of supernatant and mix. Extract with 1 volume of TE-saturated phenol/chloroform (pg. 140). Vortex for 1 minute and spin for 10 minutes at 10,000 x g.
10. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as above.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

(continued)

11. Transfer the upper, aqueous phase to a fresh tube.

DNA isolated from a liquid lysate.

a. Add 0.5 volume of 7.5M ammonium acetate and mix.

b. Add 2 volumes (volume after Step a) of 100% ethanol and mix. Place at 0°C for 10-15 minutes.

c. Centrifuge at 14,000 x g for 10 minutes. Carefully remove the supernatant.

d. Resuspend the pellet in 1ml of 70% ethanol and place at –70°C for 10-15 minutes. Centrifuge at 14,000 x g for 5 minutes.

e. Carefully remove the supernatant and dry the DNA pellet under vacuum.

f. Resuspend the pellet in TE buffer (pg. 140). Let the DNA dissolve overnight at 4°C. For long term storage, store at 4°C.

DNA isolated from a plate lysate.

a. Add 0.5 volume of 5M NaCl.

b. Add 0.33 volume (volume after Step a.) of 30% PEG-8000 in 1.5M NaCl. Mix well and place on ice for 30 minutes.

c. Centrifuge at 12,000 x g for 5 minutes. Carefully remove and discard the supernatant. The DNA pellet appears as a gelatinous disk.

d. Add 1ml of 70% ethanol, vortex gently and centrifuge at 12,000 x g for 5 minutes to precipitate the DNA and remove excess PEG. **Note:** This ethanol wash may be repeated to ensure complete removal of PEG, but this is usually not necessary.

e. Carefully remove and discard the supernatant. Dry the pellet which now appears white.

f. Resuspend the pellet in TE buffer. Let the DNA dissolve overnight at 4°C. For long term storage, store at –20°C.

12. If the isolated lambda DNA is to be sequenced, it is necessary to treat the sample with RNase A beforehand. This treatment will minimize problems due to nonspecific background bands. A protocol for RNase A treatment is provided below:

a. Add DNase-free RNase A (pg. 140) to a final concentration of 100μg/ml.

b. Incubate at 37°C for 30 minutes.

c. Add NaCl to 50mM. Extract with 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and spin in a microcentrifuge (12,000 x g) for 5 minutes.

d. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and spin in a microcentrifuge for 5 minutes.

e. Transfer the upper, aqueous phase to a fresh tube.

f. Add 2 volumes of 100% ethanol, mix and place at –20°C for 30 minutes.

g. Spin for 10 minutes in a microcentrifuge. Carefully remove the supernatant.

h. Wash the pellet in 1ml of 70% ethanol. Spin for 5 minutes in a microcentrifuge.

i. Carefully remove the supernatant and dry the DNA pellet under vacuum.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

(continued)

D. Mini-Prep Isolation of Lambda DNA

Reagents Needed

- RNase A
- DNase I
- phage precipitation solution
- phage buffer (pg. 141)
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- isopropanol
- ethanol (70%)
- TE buffer

Protocol

1. Add RNase A and DNase I to the lambda lysate supernatant, each to a final concentration of 1µg/ml. Incubate for 30 minutes at 37°C.

2. Add an equal volume of phage precipitation solution (below) and incubate for 1 hour at 0°C (ice water).

3. Recover the precipitated phage particles by centrifugation at 10,000 x g for 20 minutes at 4°C.

4. Remove the supernatant by aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away.

5. Add 1ml of phage buffer (pg. 141) per 10ml of initial lysate (Step D.1) and resuspend the phage particles by gentle vortexing.

6. Centrifuge at 8,000 x g for 2 minutes at 4°C to remove debris.

7. Extract once with 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

8. Transfer the upper, aqueous phase to a fresh tube. Extract once with 1 volume of TEN-saturated phenol/chloroform. Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

9. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as above.

10. Transfer the upper, aqueous phase to a fresh tube. Add an equal volume of isopropanol. Mix and leave at -70°C for 20 minutes. Centrifuge at 12,000 x g for 10 minutes at 4°C.

11. Carefully drain the supernatant. Wash the pellet with 1ml of 70% ethanol. Dry the pellet under vacuum and resuspend it in 50-100µl of TE buffer. Store at 4°C. From 2×10^{11} pfu, one should obtain between 5-10µg of recombinant lambda DNA.

Note: This mini-prep protocol may not remove all contaminating RNA. If desired, the purified DNA may be treated with 100µg/ml DNase-free RNase A (below) for 30 minutes at 37°C followed by phenol and chloroform extractions and ethanol precipitation (see pg. 139, step C.12).

Composition of Solutions

SM buffer (per liter):

50mM	Tris-HCl, pH 7.5
100mM	NaCl
8mM	$MgSO_4$
0.01%	gelatin (5ml of a 2% stock)

Sterilize by autoclaving. Store in 50ml aliquots.

Phage precipitation solution:

20%	(w/v) polyethylene glycol (MW 8,000)
2M	NaCl

TE buffer:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TEN buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

DNase-free RNase A:

To make DNase-free RNase A, prepare a 10mg/ml solution of RNase A in 10mM Tris-HCl, pH 7.5, 15mM NaCl. Heat at 100°C for 15 minutes and cool slowly to room temperature.

V. Preparation of Lambda Lysates and Isolation of Lambda DNA

(continued)

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust pH to 7.5 with NaOH and autoclave.

LB plates:

Add 15 grams of Bacto-agar to one liter of LB medium. Autoclave. Pour 30-35ml of medium into 100mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

LB top agarose (100ml):

1g	Bacto-tryptone
0.5g	Bacto-yeast Extract
0.5g	NaCl
0.6g	agarose

Autoclave. When the solution has cooled add 1ml of 1M $MgSO_4$.

Phage buffer:

20mM	Tris-HCl, pH 7.4
100mM	NaCl
10mM	$MgSO_4$

Release buffer:

10mM	Tris-HCl, pH 7.8
10mM	EDTA

VI. Random Primer Labeling of DNA

The Prime-a-Gene® labeling system is based on the method developed by Feinberg and Vogelstein (19,20), in which a mixture of random hexanucleotides is used to prime DNA synthesis *in vitro* from any linear double-stranded template DNA. With this straightforward method, it is possible to generate probes of extremely high specific activity (routinely $>10^9$ cpm/µg), even using DNA fragments directly cut from agarose gels. Since the input DNA serves as a template and remains intact during the reaction, minimal amounts of DNA (25ng) can be labeled to a high specific activity. Typically, greater than 70% of the labeled deoxyribonucleotide can be incorporated. The resulting probes frequently are longer than nick translated probes and can be greater than 50% of the length of the original template (21-26).

A. Protocol for Random Primer Labeling

Solutions Required

- TE buffer (pg. 145)
- labeling buffer (pg. 145, supplied)
- dNTPs, labeled and unlabeled (see Notes 1 and 3, pg.142)

The following reaction conditions are optimized for labeling 25ng of DNA template. If other amounts of DNA are to be labeled, the reaction volume should be adjusted up or down in proportion. Using larger amounts of DNA in the standard 50µl volume will result in lower specific activities and shorter average probe lengths. The use of smaller amounts results in a slower reaction. For example, whereas the standard reaction with 25ng of DNA is essentially complete in 60 minutes, a reaction with 10ng of DNA in 50µl may take as long as 5 hours to go to completion. If necessary, the reaction can be allowed to proceed overnight at room temperature.

Protocol

1. Allow the system components to thaw on ice. Be careful to store the Klenow enzyme at –20°C and return it to the freezer immediately after using.
2. Dissolve the DNA in sterile H_2O or TE buffer (pg. 145) at 1-25µg/ml and denature the sample by heating it in a screw-capped microcentrifuge tube at 95-100°C for 2 minutes. Rapidly chill the tube in an ice bath. Assemble the reaction in a separate microcentrifuge tube, on ice, by the addition of the following reagents in the order listed:

VI. Random Primer Labeling of DNA

(continued)

Component		Final Concentration
labeling 5X buffer (pg. 145)	10µl	
mixture of the unlabeled dNTPs (see Note 1)	2µl	20µM each
denatured DNA template (see Note 2)	25ng	500ng/ml
10mg/ml acetylated (nuclease-free) BSA	2µl	400µg/ml
[α-^{32}P]dNTP (50µCi, 3,000 Ci/mmole) (see Note 3)	5µl	333nM
Klenow enzyme	5u	100u/ml
sterile H_2O to final volume	50µl	

3. Mix gently and incubate the reaction tube at room temperature for 60 minutes.

4. Terminate the reaction by heating at 95-100°C for 2 minutes and subsequently chilling in an ice bath. Add EDTA to 20mM and use directly in a hybridization reaction or store at –20°C for later use.

Notes:

1. To prepare the unlabeled dNTPs, mix 1µl each of the three nonisotopically labeled dNTPs (1.5mM stock solutions) to yield a 3µl solution at 500µM for each dNTP. Also see Note 3, below.

2. The DNA template should be linear and can be in molten agarose (20). It is usually best to radiolabel only the insert DNA, rather than the entire vector. This considerably increases the signal to noise ratio of the resultant probe (19,20).

3. Although [α-^{32}P]dCTP is used in the standard reaction, optimum labeling can be achieved using a variety of isotopes (^{3}H, ^{35}S, ^{32}P, ^{125}I). To achieve an optimum probe length, the amount of radiolabeled dNTP should be 10-125pmoles. However, the highest incorporation efficiency occurs when 30pmoles of a labeled dNTP are present. A high incorporation efficiency becomes important when the background contributed by unincorporated label is an issue. The final specific activity of the DNA is influenced by two factors: the specific activity of the labeled dNTP (Ci/mmole) and how many of the 4 dNTPs (at 10-125pmole each) in a reaction are radiolabeled. The use of multiple labeled dNTPs should be considered when using [^{35}S] or [^{3}H].

The volume of aqueous labeled dNTP should not exceed 50% of the total reaction volume. Labeled dNTPs supplied in 50% ethanol must be evaporated to dryness and redissolved in H_2O before use in the reaction.

B. Removal of Unincorporated Label (Optional)

This step is not necessary unless incorporation levels are low. Unincorporated labeled nucleotides can be removed by size exclusion chromatography on Sephadex G-50 spin columns (17) or by selective precipitation of the labeled DNA with spermine or with ammonium acetate and ethanol (described below).

1. Sephadex G-50 Spin Columns

Sephadex G-50 spin columns (17) have the advantages that they yield probe virtually free of unincorporated dNTPs and also substantially reduce the content of DNA oligomers less than 70 bases in length. This is useful when generating hybridization probes, since optimal signal to noise ratios are achieved with larger probes (500-1,500 bases in length) (27). A protocol for the use of Sephacryl S-400 spin columns is provided in the cDNA Synthesis and Cloning chapter, pg. 214.

VI. Random Primer Labeling of DNA

(continued)

2. Selective Precipitation of DNA with Spermine

Solutions Required

- TE buffer (pg. 145)
- 10mg/ml herring sperm DNA
- 0.1M spermine·4HCl
- 5M NaCl

Protocol

a. Add 140µl of TE buffer and 4µl of a stock solution of herring sperm DNA (10mg/ml) to the 50µl reaction. Mix.

b. Add 4µl of 0.1M spermine·4HCl, mix, and chill on ice for 15 minutes.

c. Spin for 10 minutes in a microcentrifuge (12,000 x g) at 4°C.

d. Carefully remove the supernatant with a pipet. Rinse the pellet with 400µl of a solution containing 396µl of TE buffer and 4µl of spermine·4HCl. Vortex briefly and spin for 2 minutes at 4°C in a microcentrifuge.

e. Carefully remove the supernatant and resuspend the pellet in 520µl of TE buffer. Add 40µl of 5M NaCl, mix, and warm to about 55°C for 15-30 minutes to ensure that the pellet is completely dissolved.

3. Selective Precipitation of DNA with Ammonium Acetate/Ethanol

Selective precipitation of labeled DNA with ammonium acetate and ethanol results in the precipitation of DNA >20 nucleotides in length, while free dNTPs remain in the supernatant. The recovery of labeled DNA by this method depends on the length and concentration of the DNA, but can be as low as 50%.

Solutions Required

- 4M ammonium acetate, pH 4.5
- ethanol
- TE buffer (pg. 145)

Protocol

a. Add 1 volume of 4M ammonium acetate, pH 4.5, and vortex.

b. Add 2 volumes of ethanol (1 volume = total volume in Step a), mix, and chill in an ice bath for 15 minutes.

c. Heat at 37°C for 2 minutes with occasional gentle mixing. This step redissolves free deoxyribonucleotides precipitated in Step b.

d. Centrifuge at 12,000 x g for 15 minutes and carefully aspirate the supernatant.

e. Wash the pellet once in 0.5ml of 0.67M ammonium acetate, pH 4.5, 67% ethanol at room temperature with gentle shaking. Centrifuge and carefully aspirate as before.

f. Wash the pellet once in 90% ethanol and dry under vacuum.

g. Redissolve the labeled DNA in TE buffer and use for probe hybridization.

C. Determination of Percent Incorporation

The percent of label incorporated may be determined either by a DE81 filter-binding assay or by TCA precipitation. These two methods are described below.

1. DE81 Filter-Binding Assay

Solutions Required

- 0.2M EDTA
- 0.5M sodium phosphate, pH 6.8 (pg. 145)

Protocol

a. Dilute 1µl of the labeling reaction 1:100 in 0.2M EDTA. Spot 3µl, in duplicate, of the diluted sample on Whatman DE81 2.3cm circular filters.

b. Dry the filters briefly under a heat lamp. One filter is kept aside and is used directly for the determination of total cpm in the sample.

c. Wash the other filter in 50ml of 0.5M sodium phosphate, pH 6.8 twice for 5 minutes to remove the unincorporated cpms.

d. Dry the washed filter under a heat lamp.

e. Add the appropriate scintillation fluid to each filter and count in a scintillation counter.

Nucleic Acid Detection, Purification and Labeling

VI. Random Primer Labeling of DNA

(continued)

2. TCA Precipitation

Solutions Required

- 0.2M EDTA
- 10% trichloroacetic acid, 1% sodium pyrophosphate
- 10% trichloroacetic acid
- carrier DNA or acetylated BSA
- ethanol

Protocol

a. Dilute 1µl of the labeling reaction 1:100 in 0.2M EDTA. Spot 3µl of this diluted sample on a glass fiber or nitrocellulose filter for determination of the total cpm in the sample. Let the filter air dry.

b. Transfer 3µl of the same dilution to a tube containing 100µl of 0.1mg/ml carrier DNA or acetylated BSA and 20mM EDTA. Mix well.

c. Add 1.3ml of ice-cold 10% trichloroacetic acid (TCA), 1% sodium pyrophosphate to the mixture and leave on ice for 20 minutes.

d. Collect the precipitated DNA by vacuum filtration on a glass fiber or nitrocellulose filter. Wash the filter at least 3 times with 5ml of cold 10% TCA followed by a brief rinse with acetone (glass fiber only) or 95% ethanol and let air dry.

e. Add the appropriate scintillation fluid to each filter and count in a scintillation counter.

Note: It is not necessary to use scintillation fluid for counting ^{32}P-labeled samples. The Cerenkov radiation emitted from such samples can be detected by a scintillation counter set to monitor the tritium window. Although the absolute number of counts is not the same between the two methods (because Cerenkov counting is less than half as efficient), they will be in proportion from sample to sample.

D. Calculation of Specific Activity

1. Random primer labeling results in net DNA synthesis. To calculate the specific activity of the labeled probe DNA, it is necessary to first calculate the amount of DNA generated in the reaction had there been 100% incorporation:

$$\frac{\mu\text{Ci dNTP added} \times 4 \times 330\text{ng/nmole}}{\text{specific activity dNTP (Ci/mmole=}\mu\text{Ci/nmole)}} = \text{ng theoretical yield}$$

2. Next, calculate the percent incorporation from the TCA precipitation results:

$$\frac{\text{cpm incorporated} \times 100\%}{\text{total cpm}} = \text{\% incorporation}$$

3. Determine the amount of DNA synthesized:

$$\text{\% incorporation} \times 0.01 \times \text{theoretical yield} = \text{ng DNA synthesized}$$

4. Calculate the specific activity of the product:

$$\frac{\text{total cpm incorp. (cpm incorp.} \times 33.3 \times 50)}{(\text{ng DNA synth.} + \text{ng input DNA}) \times 0.001\mu\text{g/ng}} = \text{cpm/}\mu\text{g}$$

Note: The factors 33.3 and 50 are derived from using 3µl of a 1:100 dilution for TCA precipitation and having to convert this back to a 50µl total reaction volume.

Example

Using 50µCi [α-^{32}P]dCTP (3,000Ci/mmole) in a standard reaction, the calculation is as follows:

$$\frac{50\mu\text{Ci} \times 4 \times 330\text{ng/nmole}}{3{,}000\mu\text{Ci/nmole}} = 22\text{ng theoretical yield}$$

Assuming that 4.92×10^4cpm were TCA precipitated and that the unprecipitated sample had 5.28×10^4cpm:

$$\frac{4.92 \times 10^4 \times 100\%}{5.28 \times 10^4} = 93\% \text{ incorporation}$$

$$0.93 \times 22\text{ng} = 20.5\text{ng DNA synthesized}$$

The specific activity is therefore:

$$\frac{4.92 \times 10^4\text{cpm} \times 33.3 \times 50}{(20.5\text{ng} + 25\text{ng}) \times 0.001\mu\text{g/ng}} = 1.8 \times 10^9 \text{ cpm/}\mu\text{g}$$

VI. Random Primer Labeling of DNA

(continued)

E. Troubleshooting

All components of the Prime-a-Gene labeling system are tested to ensure maximum performance. In the event that poor results are obtained, the following points should be considered:

1. Check that the procedure was followed as specified and that the DNA was denatured prior to beginning the reaction.

2. Control lambda DNA has been included so that the reaction kinetics can be verified. This DNA must be denatured before use in random primer labeling. Several time points can be taken to monitor the progress of the reaction.

3. Possible problems with the labeled dNTP include losses during manipulation of reagents supplied in aqueous ethanol and quenching of counts by moisture retained in sample filters.

4. High background during hybridization can result either from excessive unincorporated [α-^{32}P]dNTP or too much template DNA in the reaction (this yields shorter probes which have a reduced hybridization specificity).

Composition of Solutions

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

Labeling 5X buffer:

250mM	Tris-HCl, pH 8.0
25mM	$MgCl_2$
10mM	DTT
1mM	HEPES, pH 6.6
26	A_{260} units/ml random hexadeoxyribonucleotides

0.5M Sodium phosphate, pH 6.8 (per liter):

47.25g	NaH_2PO_4
22.35g	Na_2HPO_4

*Buffer tablets available from Promega.

VII. Nick Translation Labeling of DNA

The nick translation system has been designed for the efficient incorporation of labeled deoxynucleotide triphosphates into duplex DNA. Free 3′ hydroxyl ends (nicks) are created within the unlabeled DNA by DNase I. DNA polymerase I then catalyzes the addition of a nucleotide residue to the 3′-hydroxyl terminus of the nick. At the same time, the 5′→ 3′ exonuclease activity of this enzyme removes the nucleotide from the 5′-phosphoryl terminus of the nick. A new nucleotide with a 3′-OH group is incorporated at the position where the original nucleotide was excised, and the nick is thus shifted along by one nucleotide in a 3′ direction. This 3′ shift of the nick results in the sequential addition of radioactively labeled nucleotides to the DNA while the pre-existing nucleotides are removed (17). DNA probes prepared by nick translation can be used for a wide variety of hybridization techniques such as gel blots and colony plaque lifts. Using the protocol provided, typically greater than 65% of the labeled deoxyribonucleotide is incorporated, generating probes of high specific activity (routinely 10^8cpm/μg). Highly purified DNA polymerase I and DNase I are provided in a stable enzyme mix, assuring synthesis of the optimum probe size distribution. Separate deoxynucleotide solutions and buffers supplied with the system offer convenience and flexibility.

A. Standard Reaction

Solutions Required

- unlabeled dNTPs (supplied, see Note 1)
- [α-^{32}P]dCTP (400Ci/mmole)
- nick translation 10X buffer (pg. 146, supplied)
- optimized enzyme mix (supplied)
- stop solution (pg. 146, supplied)

Protocol

1. Mix the following components:

nucleotide mix (supplied, see Note 1)	10μl
nick translation 10X buffer	5μl
sample DNA in H_2O	1μg
[α-^{32}P]dCTP (70μCi at 400Ci/mmole and 10mCi/ml) (see Note 2)	7μl
optimized enzyme mix	5μl
sterile H_2O (see Note 3)	to final volume 50μl

2. Incubate at 15°C for 60 minutes (see Note 4).

3. Add 5μl of the stop solution provided.

Nucleic Acid Detection, Purification and Labeling

VII. Nick Translation Labeling of DNA

(continued)

B. Determination of Percent Incorporation and Specific Activity

The percent of label incorporated may be determined either by a DE81 filter-binding assay or by TCA precipitation. These two methods are described in Section VI.C on pg. 143. The specific activity of the labeled DNA may be calculated using the equations provided below.

Calculate the percent incorporation.

$$\frac{\text{cpm incorporated} \times 100}{\text{total cpm}} = \text{percent incorporation}$$

Calculate the specific radioactivity of the product.

$$\frac{\text{cpm incorporated} \times 33.3 \times 50}{\text{mg input DNA}} = \text{cpm/}\mu\text{g}$$

Note: The factors 33.3 and 50 are derived from using 3µl of a 1:100 dilution and having to convert this back to a 50µl total reaction volume.

Example: Assuming that 70µCi of labeled dNTP were added to the reaction and that 6.19×10^4 cpm were incorporated into the diluted sample, the calculations are as follows:

$$\frac{6.19 \times 10^4 \text{cpm} \times 100}{9.24 \times 10^4 \text{cpm}} = 67\% \text{ incorporation}$$

$$\frac{6.19 \times 10^4 \times 33.3 \times 50}{1\text{mg DNA}} = 1.03 \times 10^8 \text{cpm/}\mu\text{g DNA}$$

Notes:

1. The nucleotide mix is prepared by mixing equal volumes of the 3 unlabeled nucleotides (1.5mM stock solutions) chosen minus the nucleotide selected as label.

2. To produce probes with higher specific activities, a second labeled nucleotide can be used in place of one of the unlabeled nucleotides. The final concentration of any labeled nucleotide should not fall below 3µM.

3. The reaction volume may be scaled between 10-100µl, but the components should be kept in the same proportions as in the standard reaction.

4. It is important to maintain the reaction temperature at 15°C. Higher temperatures can produce "snapback" DNA structures which lower hybridization efficiency (17). In general, longer incubation times result in greater incorporation of label but tend to slightly reduce the overall length of the labeled fragment.

5. Unincorporated dNTPs can be removed either by chromatography or centrifugation using a small column of Sephadex G-50.

Composition of Solutions

Nick translation 10X buffer:

500mM	Tris-HCl, pH 7.2
100mM	$MgSO_4$
1mM	DTT

Stop solution:

0.2M	EDTA, pH 8.0

VIII. Synthesis of [γ-^{32}P] Nucleotides with the GammaPrep®-A System

The GammaPrep-A system is a single tube reaction mixture which synthesizes [γ-^{32}P]ATP. ^{32}P-labeled ATP made with the GammaPrep-A system can be used directly in DNA labeling reactions with T4 polynucleotide kinase. The end-labeled DNA can then be used in sequencing reactions. [γ-^{32}P]ATP can also be used in the synthesis of [5′-^{32}P]pCp.

The system uses a modification of the method of Walseth and Johnson (28). It contains all the necessary enzymes and substrates in a single tube, except for the ^{32}P. There are 5nmole of ADP in the 50µl reaction mixture. Addition of 2mCi of carrier-free ^{32}P to the GammaPrep-A reaction tube followed by a 45 minute incubation at room temperature results in virtually 100% incorporation of the ^{32}P into [γ-^{32}P]ATP. The reaction is terminated simply by heating. System reaction vials each yield up to 2mCi of [γ-^{32}P]NTP at a specific activity of 3,000-6,000Ci/mmole.

VIII. Synthesis of [γ-^{32}P] Nucleotides with the GammaPrep®-A System

(continued)

Procedure

1. Remove a reaction tube from the freezer (–70°C) and thaw at room temperature. (Do not allow the solution to thaw longer than necessary). The volume of the reaction mixture is 50µl.
2. Add an additional 50µl volume to the reaction tube consisting of up to 2mCi of ^{32}P (50µl of ^{32}P at 40mCi/ml) plus sterile water, if necessary.
3. Vortex gently but thoroughly and incubate for 45 minutes at room temperature (20-22°C).
4. Stop the reaction by heating the contents of the tube for 5 minutes at 90°C. Cool on ice.
5. Quick-freeze the solution (after dispensing into aliquots, if desired) in a dry ice/ethanol bath and store frozen at –70°C in a plexiglass container.
6. The [γ-^{32}P]ATP can now be used directly for the desired application.

IX. 3′-End Labeling to Fill Recessed 3′ Ends of Double-Stranded DNA

This procedure is used to fill and label recessed 3′ ends of double-stranded DNA following cleavage with a restriction enzyme (17).

Solutions Required

- Klenow 5X buffer
- 1mg/ml acetylated BSA
- 2mM unlabeled dNTPs
- [α-^{32}P]dNTP (400Ci/mmole)
- TE-saturated phenol/chloroform
- 2M NaCl
- ethanol
- TE buffer

Protocol

1. Mix the following:

digested DNA	1µg
Klenow 5X buffer	10µl
1mg/ml acetylated BSA (optional)	1µl
2mM unlabeled dNTPs (as needed)	1µl
appropriate [α-^{32}P]dNTP (400Ci/mmole, 1mCi/ml)	2µl
Klenow DNA polymerase	1-5u
sterile H_2O	to final volume 50µl

2. Incubate at room temperature for 10 minutes.
3. Add 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute. Centrifuge at 12,000 x g for 5 minutes.
4. Transfer the aqueous layer to a fresh tube, add 0.1 volume of 2M NaCl, and mix.
5. Add 2 volumes of ethanol. Incubate at –20°C for 30 minutes.
6. Centrifuge at 12,000 x g for 10 minutes.
7. Remove and discard the supernatant. Dry the pellet under vacuum.
8. Redissolve the DNA in 50µl of TE buffer.

Composition of Solutions

Klenow 5X buffer:

0.25M	Tris-HCl, pH 7.2
50mM	$MgSO_4$
0.5mM	DTT

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

*Buffer tablets available from Promega.

Nucleic Acid Detection, Purification and Labeling

X. 3′-End Labeling With Terminal Transferase

Terminal deoxynucleotidyl transferase is an enzyme that catalyzes the repetitive addition of mononucleotides from a dNTP to the terminal 3′-OH of a DNA initiator, accompanied by the release of inorganic phosphate (29). The enzyme thus provides a unique method for the labeling of the 3′ termini of DNA with ^{32}P for subsequent utilization in hybridization assays.

Two different protocols for the incorporation of ^{32}P are provided. The first illustrates the addition of a [α-^{32}P]dNTP "tail" to the 3′ termini of single-stranded DNA fragments. Incorporation can also be limited to a single nucleotide by using [α-^{32}P]cordycepin-5′-triphosphate (30). This analog lacks a free 3′ hydroxyl group, preventing incorporation of additional nucleotides.

Promega's 3′-end labeling system includes a positive control DNA substrate consisting of an oligo$(dT)_{12}$ primer. The oligo$(dT)_{12}$ can be used to monitor incorporation rates. Once labeled, it can also be used to optimize hybridization conditions by acting as a probe for the presence of poly(A) sequences in the sample.

Reagents to be Supplied by the User

- TBE 10X buffer (pg. 150)

A. Addition of [α-^{32}P]dNTP "Tails" to 3′ Termini of Single-Stranded DNA Primers

1. Set up the following standard reaction:

terminal transferase 5X buffer (supplied, pg. 150)	4.0µl
primer (corresponds to approximately 1µl of provided positive control)	2pmoles
[α-^{32}P]dATP (800Ci/mmole, 10mCi/ml)	1.6µl
terminal transferase (at 10-20u/µl)	1.0µl
H_2O	to final volume 20µl

2. Incubate at 37°C for 30-60 minutes.

3. Stop the reaction by heating at 70°C for 10 minutes.

Notes:

1. The ratio of labeled nucleotide to DNA primer determines approximately the number of nucleotides added to the 3′ termini. As an example, 10pmoles of labeled nucleotide will add a tail of 10 nucleotides to 1pmole of DNA primer. Additions of this nature generate DNA probes with specific activities greater than 1×10^9cpm/µg.

2. The enzyme to substrate ratio is also critical for obtaining uniform addition of labeled nucleotides. Therefore, 10-20 units of enzyme are recommended for 2-4pmoles of substrate DNA. Ratios lower than this produce probes of varying lengths and reduce incorporation rates.

3. Reactions can be carried out overnight without affecting the quality of the end product.

4. Enzyme activity is inhibited if the amount of label present in the reaction exceeds 40% of the total reaction volume. Under these conditions, enzyme activity is inhibited. To avoid this problem, reactions can be scaled up accordingly.

5. In the presence of Co^{++} (in terminal transferase 5X buffer), double-stranded DNA will also be labeled. DNA with a protruding 3′ terminus is preferred. Blunt-ended or recessed 3′ termini will also be labeled, although not with uniform efficiency.

Table 1. Amount of DNA Primer (ng) Needed to Equal 2pmoles.

Primer Length	ng of primer equal to 2pmoles
15mer	10ng
18mer	12ng
24mer	16ng
31mer	21ng

X. 3′-End Labeling With Terminal Transferase

(continued)

B. Addition of [α-^{32}P]Cordycepin-5′-Triphosphate to 3′ Termini of a Single-Stranded DNA Primer

1. Set up the following sample reaction:

terminal transferase 5X buffer	10µl
DNA substrate (corresponds to approximately 5µl of provided positive control)	10pmoles
[α-^{32}P]cordycepin-5′-triphosphate (at 3,000Ci/mmole, 10mCi/ml)	7.5µl
terminal transferase (at 10-20u/µl)	2µl
H_2O	to final volume 50µl

2. Incubate at 30 minutes at 37°C for 10 minutes.
3. Stop the reaction by heating at 70°C for 10 minutes.

Notes:

1. The ratio of label to primer should be at least 1.5:1 to 2.0:1 in order for a successful reaction to occur.
2. In the presence of Co^{++} (in terminal transferase 5X buffer), double-stranded DNA will also be labeled. DNA with a protruding 3′ terminus is preferred. Blunt-ended or recessed 3′ termini will also be labeled, although not with uniform efficiency.

C. Determination of Percent Incorporation and Specific Activity

The percent of label incorporated may be determined either by a DE81 filter-binding assay or by TCA precipitation. These two methods are described in Section VI.C on pg. 143. The specific activity of the labeled DNA may be calculated using the equations provided below.

Calculate the percent incorporation:

$$\frac{\text{cpm incorporated} \times 100}{\text{total cpm}} = \text{percent incorporation}$$

Calculate the specific radioactivity of the product:

$$\frac{\text{\% incorporation} \times \text{total cpm added to reaction}}{\text{mg of DNA substrate in reaction}} = \text{specific activity of probe}$$

Example:

$$\frac{48{,}500 \times 100}{51{,}755} = 94\% \text{ incorporation}$$

$$\frac{0.94 \times 3.5 \times 10^7 \text{cpm}}{008\text{mg}} = 4 \times 10^9 \text{cpm/µg DNA}$$

X. 3′-End Labeling With Terminal Transferase

(continued)

D. Gel Analysis

A convenient method to observe if a "tail" has been added to the DNA substrate is by analysis on a denaturing polyacrylamide gel. Typically, high percentage gels (10-20%) are needed to distinguish short additions. A small sample of the diluted reaction can be analyzed in such a manner.

Composition of Solutions

Terminal transferase 5X buffer:

500mM	cacodylate, pH 6.8
1mM	$CoCl_2$
0.5mM	DTT
500µg/ml	BSA

TBE 10X buffer (for gel analysis):

0.89M	Tris base
0.89M	boric acid
20mM	EDTA

Table 2. Components of Sequencing Gel Solutions (sufficient for 100ml).

Gel %	Acrylamide	Bis Acrylamide	Urea	TBE 10X Buffer	Deionized H_2O	10% AP*	TEMED
10%	9.5g	0.5g	48g	10ml	40ml	500µl	50µl
12%	11.4g	0.6g	48g	10ml	40ml	500µl	50µl
16%	15.2g	0.8g	48g	10ml	40ml	500µl	50µl
20%	19.0g	1.0g	48g	10ml	40ml	500µl	50µl

*10% ammonium persulfate should be made fresh weekly in deionized H_2O and stored at room temperature.

XI. 5′-End Labeling Using T4 Polynucleotide Kinase

This procedure is useful for radioactive end labeling of oligonucleotides (17). The kinase reaction requires that the 5′ end has been previously dephosphorylated with alkaline phosphatase. The alkaline phosphatase treatment described below can also be used to prevent recircularization and religation of linearized cloning vehicle DNA by removing phosphate groups from both 5′ termini.

Promega's 5′-end labeling system includes T4 polynucleotide kinase, calf intestinal alkaline phosphatase and buffers for their use. An oligonucleotide and the pGEM® DNA markers are also included as controls for the efficiency of the reaction. The pGEM DNA markers also provide a convenient range of fragments for analysis of kinased samples by gel electrophoresis.

Reagents to be Supplied by the User

- TE-saturated phenol/chloroform (pg. 152)
- 2M NaCl
- ethanol
- 0.5M EDTA
- 7.5M ammonium acetate
- TE buffer (pg. 152)

Protocol

A. Dephosphorylation Reaction

1. Prepare the following reaction to remove the phosphate groups from both 5′ termini of a linear molecule.

CIAP 10X buffer (pg. 152)	5µl
substrate DNA (up to a total of 10pmoles of 5′ ends*) or pGEM markers (1µl=1µg)	1µl
calf intestinal alkaline phosphatase (diluted in CIAP 1X buffer)	0.5u
sterile H_2O	to final volume 50µl

***Example:** 1pmole of 5′ ends of linear pBR322 DNA (4,361bp) is equivalent to 1.6µg.

a. **For protruding 5′-termini dephosphorylation (and pGEM markers).** Incubate at 37°C for 30 minutes. Add another 0.1 units of alkaline phosphatase and incubate for another 30 minutes at 37°C.

b. **For recessed 5′-termini or blunt end dephosphorylation.** Incubate at 37°C for 15 minutes, then at 56°C for 15 minutes. Add another 0.1 units of alkaline phosphatase and repeat incubations at both temperatures.

Technical Services:
800-356-9526

Nucleic Acid Detection, Purification and Labeling

XI. 5′-End Labeling Using T4 Polynucleotide Kinase

(continued)

2. To stop the reaction, add 1 volume of TE-saturated phenol/chloroform (pg. 152). Vortex for 1 minute and spin in a microcentrifuge at 12,000 x g for 2 minutes.

3. Remove the upper, aqueous phase to a fresh tube and repeat Step 2.

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and spin in a microcentrifuge as in Step 2.

5. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 2M NaCl (see Note 1).

6. Add 2 volumes of ethanol. Mix and place at –70°C for 30 minutes. Spin in a microcentrifuge for 5 minutes.

7. Carefully pour off the supernatant and dry the pellet under vacuum. Resuspend the DNA in 34μl of forward exchange 1X buffer (pg. 152).

B. Kinase Reaction

After the 5′ phosphate groups have been removed, the substrate DNA can be labeled.

1. To the reaction add the following:

[γ-^{32}P]ATP (3,000Ci/mmole at 10mCi/ml) (see Note 2, below)	15μl
T4 polynucleotide kinase (at 8-10u/μl)	1μl
final volume	50μl

2. Incubate at 37°C for 10 minutes.

3. Stop the reaction by adding 2μl of 0.5M EDTA.

4. Add 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and spin in a microcentrifuge at 12,000 x g for 2 minutes.

5. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate.

6. Add 2 volumes of ethanol (the volume after Step 5). Mix and place at –70°C for 30 minutes. Spin in a microcentrifuge for 5 minutes.

7. Redissolve the DNA in 50μl of TE buffer (pg. 152).

Notes:

1. Ammonium ions are strong inhibitors of bacteriophage T4 polynucleotide kinase; therefore, DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with kinase.

2. The concentration of ATP in the reaction should be at least 1μM.

3. The oligonucleotide control DNA provided can be labeled directly as described in the following procedure.

   a. Mix the following:

oligonucleotide (100ng)	5μl
[γ-^{32}P]ATP (3,000Ci/mmole at 10mCi/ml)	3μl
forward exchange 10X buffer	1μl
T4 polynucleotide kinase (at 8-10u/μl)	1μl
final volume	10μl

   b. Incubate at 37°C for 10 minutes.

   c. Stop the reaction by adding 1μl of 0.5M EDTA.

   Note: Phenol extraction can result in the loss of short oligonucleotides into the phenol phase. The incorporation of label into the control oligonucleotide should be determined by the DE81 filter-binding assay (pg. 143) and the unincorporated label should be removed using a G-50 column (pg. 142).

4. If the calf alkaline phosphatase is diluted in CIAP 1X buffer, it must be used immediately. If the diluted enzyme is to be stored, it should be diluted in the storage buffer.

XI. 5′-End Labeling Using T4 Polynucleotide Kinase

(continued)

C. Determination of Percent Incorporation

At this point, the incorporation may be determined directly by a DE81 filter-binding assay or by TCA precipitation (Section VI.C, pg. 144). A DE81 filter binding assay should be used to measure isotope incorporation into short oligonucleotides (≤100 bases). Procedures for separation of labeled DNA from unincorporated nucleotides are provided in Section VI. B, pg. 142.

Composition of Solutions:

CIAP (calf intestinal alkaline phosphatase) 10X buffer:

500mM	Tris-HCl, pH 9.0
10mM	$MgCl_2$
1mM	$ZnCl_2$
10mM	spermidine

Forward exchange 10X buffer:

500mM	Tris-HCl, pH 7.5
100mM	$MgCl_2$
50mM	DTT
1.0mM	spermidine

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

*Buffer tablets available from Promega.

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

XII. References

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XIII. Additional Nucleic Acid Detection, Purification and Labeling Literature Available from Promega

Technical Bulletins

002	Riboprobe® Gemini System Transcription of Cloned DNA
022	GammaPrep® Synthesis Systems: User Information
032	LambdaSorb® Phage Adsorbent
034	Alkaline Phosphatase
044	Nick Translation System
049	Prime-a-Gene® Labeling System
087	RNAgents™ Total RNA Isolation Kit
088	DNA 3′-End Labeling System
090	PolyATtract™ mRNA Isolation System
096	DNA 5′-End Labeling System

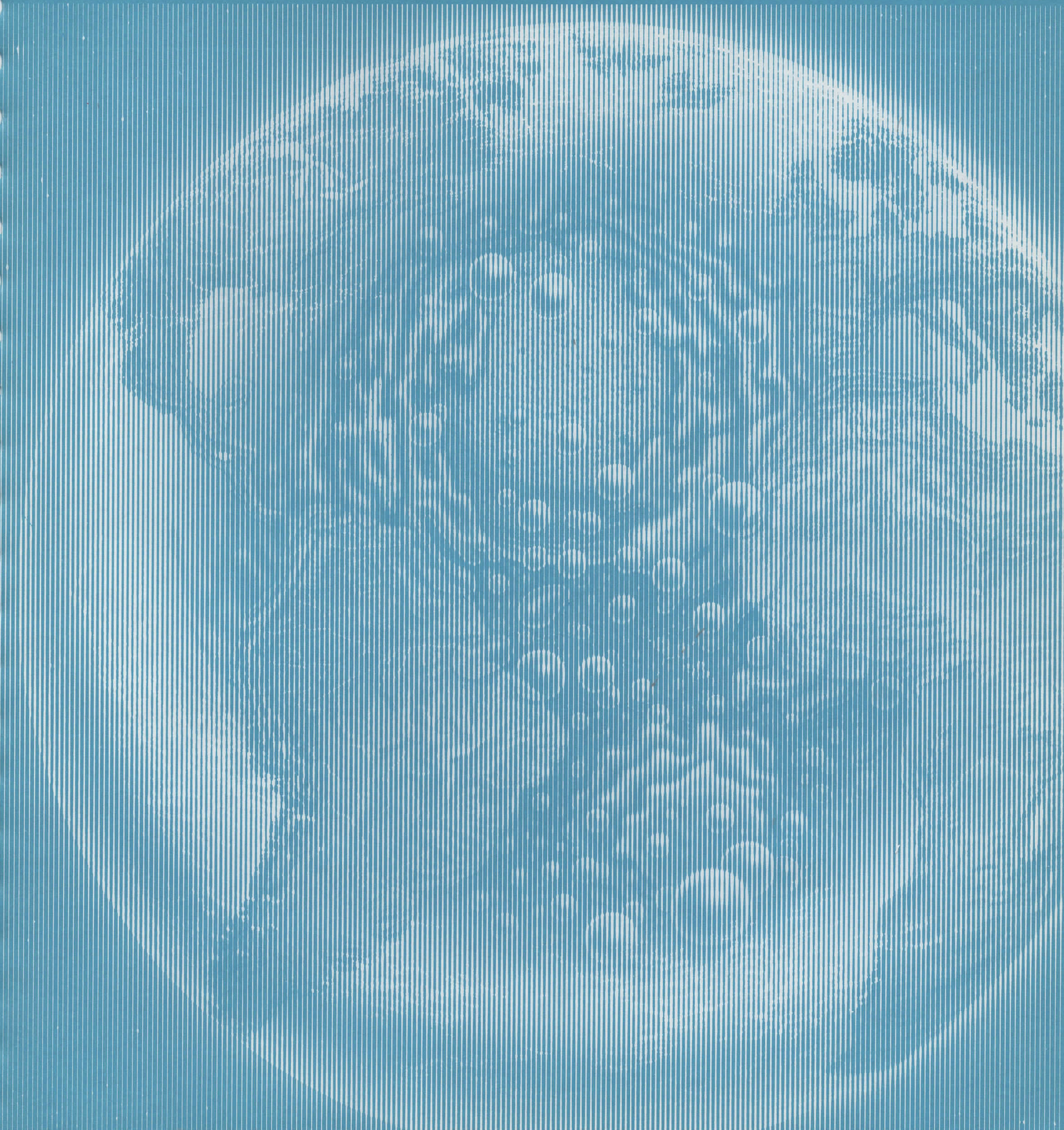
Promega Notes Articles

Issue	Title
3	High quality BSA (how to acetylate BSA)
6	Rapid isolation of lambda DNA with LambdaSorb® phage adsorbent
18	Gel electrophoresis of lambda DNA: NaCl phenomenon
19	Rapid, high yield isolation of total RNA using the RNAgents™ total RNA isolation kit
25	The PolyATtract™ magnetic mRNA isolation system: optimization and performance
26	LambdaSorb® phage adsorbent update

Nucleic Acid Detection, Purification and Labeling

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Translation *in vitro*

Contents

Translation *in vitro*

I. Introduction

A. Translation Systems

Several cell-free protein synthesizing systems have been used in recent years for the translation of mRNA isolated from various sources. Of these, Promega offers the rabbit reticulocyte lysate and the wheat germ extract. Both provide reliable, convenient, and easy to use systems to initiate translation and produce full size polypeptide products. Reticulocyte lysate is often favored for translation of larger mRNA species, and is generally recommended when microsomal membranes are to be added for cotranslational processing of translation products. Wheat germ extract readily translates certain RNA preparations, such as those containing low concentrations of double-stranded RNA (dsRNA) or oxidized thiols, which are inhibitory to reticulocyte lysate.

When appropriate, translation products may be further analyzed for cotranslational processing and core glycosylation by the addition of canine pancreatic microsomal membranes to a standard translation reaction. Processing events are generally detected as shifts in the apparent molecular weight of translation products.

Translation reactions *in vitro* may be directed by either mRNAs isolated *in vivo* or by RNA templates transcribed *in vitro* from vectors such as the Riboprobe®, Riboprobe Gemini and LambdaGEM® vectors. Procedures for the rapid isolation and poly(A)+ selection of cellular mRNAs are provided on pg. 130. When using mRNA synthesized *in vitro*, the presence of a 5′ cap structure is recommended to enhance translational activity in Promega's translational systems (1). Procedures are provided (pg. 61) for 5′ capping of such transcripts.

When large amounts (>1µg) of *in vitro* translation products are needed, the translation procedure may be modified to allow protein synthesis to occur for several hours or days (2). Contact Promega's Technical Services Department for more information on this application.

B. Coupled Transcription Translation Systems

DNA sequences cloned in plasmid vectors also may be expressed directly using Promega's *E. coli* S30 coupled transcription translation systems. These systems, prepared by modifications of the method described by Zubay (3,4), contain the components necessary for synthesis *in vitro* of either radioactively labeled or unlabeled proteins. The template DNA to be expressed must contain prokaryotic promoter sequences and ribosome binding sites. Two types of S30 extract, for supercoiled or linear DNA templates, are available (pg. 166).

II. Rabbit Reticulocyte Lysate, Nuclease Treated

A. Description

The rabbit reticulocyte translation system plays an important role in the identification of mRNA species, the characterization of their products, the investigation of transcriptional and translational control, and the cotranslational processing of secreted proteins by the addition of microsomal membranes to the translation reaction. Reticulocyte lysate is prepared from New Zealand White rabbits injected with phenylhydrazine using a standard protocol (5) which ensures reliable and consistent reticulocyte production in each lot. The reticulocytes are purified to remove contaminating cells which could otherwise alter the translational properties of the final extract. After the reticulocytes are lysed, the extract is treated with micrococcal nuclease to destroy endogenous mRNA and thus reduce background translation to a minimum. The lysate contains the cellular components necessary for protein synthesis: tRNA; rRNA; amino acids; and initiation, elongation, and termination factors. Reticulocyte lysate is further optimized for mRNA translation by adding:

- An energy generating system consisting of pretested phosphocreatine kinase and phosphocreatine.
- A mixture of tRNAs to expand the range of mRNAs which can be translated.
- Hemin to prevent inhibition of initiation.
- Potassium acetate and magnesium acetate at a level recommended for the translation of most mRNA species.

Rabbit reticulocyte lysate has been reported to contain a variety of post-translational processing activities, including acetylation, isoprenylation, proteolysis and some phosphorylation activity (6). Processing events such as signal peptide cleavage and core glycosylation can be examined by adding canine microsomal membranes to a standard translation reaction (7). See pgs. 164-165 (Canine Pancreatic Microsomal Membranes) for details.

Promega

II. Rabbit Reticulocyte Lysate, Nuclease Treated

(continued)

B. Translation Procedure

Materials to be Supplied by the User

The following reagents are not included with the rabbit reticulocyte, wheat germ or S30 systems and must be supplied by the researcher:

- double-distilled, RNase-free water
- RNasin® ribonuclease inhibitor
- isotopically labeled amino acids, typically [^{35}S]methionine, [^{35}S]cysteine, [^{3}H]leucine or [^{14}C]leucine
- 1N NaOH/2% H_2O_2 for hydrolysis of aminoacyl tRNAs. Make up 100ml and store at 4°C.
- 1N NaOH (for wheat germ or S30)
- 25% (w/v) trichloroacetic acid (TCA)/2% casamino acids for precipitation of translation products. Make up 500ml and store at 4°C.
- 5% (w/v) TCA for washing of precipitated polypeptides. Make up 500ml and store at 4°C.
- acetone
- reagents for scintillation counting and fluorography
- reagents for performing SDS gel electrophoresis (see pg. 170)

The following equipment will be needed to perform and analyze translation reactions *in vitro*:

- polypropylene microcentrifuge tubes, 0.5ml and 1.5ml capacity
- adjustable pipettors and disposable, RNase-free plastic tips
- plastic gloves
- constant temperature bath (for 25°C, 30°C, 37°C and 67°C)
- vortex mixer
- microcentrifuge
- GF/A glass fiber filters and a vacuum filtration apparatus
- scintillation counter and vials
- slab gel electrophoresis and gel drying apparatus

About 2 hours should be allowed to perform the translation reaction. The SDS gel can conveniently be set up and cast (1 hour) during this time. Allow 2 hours to perform TCA precipitation and preparation of filters for scintillation counting. Two hours should also be allowed to load and run the SDS gel, with an additional 2 hours for the optional staining and destaining procedures.

Protocol

1. Remove the reagents from storage and allow them to thaw slowly on ice.
2. Heat the template mRNA at 67°C for 10 minutes and immediately cool on ice. This increases the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure.
3. Assemble the reaction components appropriate to the label being used (see Standard Reactions, below) in a 0.5ml polypropylene microcentrifuge tube. Gently mix the lysate with the pipettor upon addition of each component. If necessary, spin briefly in a microcentrifuge to return the sample to the bottom of the tube. (**Note:** We recommend including a control reaction containing no added mRNA. This allows measurement of any background incorporation of labeled amino acids.)
4. Incubate the reticulocyte translation reactions at 30°C for 60 minutes.
5. Analyze the results of translation. Procedures are provided for incorporation assays (pg. 168) and gel analysis of translation products (pg. 170).

Examples of Standard Reactions:

[^{35}S]Methionine

nuclease treated lysate	35µl
H_2O	7µl
RNasin® ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus methionine)	1µl
RNA substrate in H_2O (see Note 2, pg. 158)	2µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	4µl
	50µl

(Final [^{35}S]methionine concentration = 0.8mCi/ml)

Translation *in vitro*

II. Rabbit Reticulocyte Lysate, Nuclease Treated

(continued)

[^{3}H]Leucine

nuclease treated lysate	35µl
H_2O	6µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus leucine)	1µl
RNA substrate in H_2O (see Note 2)	2µl
[^{3}H]leucine (100-200Ci/mmole) at 5mCi/ml	5µl
	50µl

(Final [^{3}H]leucine concentration =0.5mCi/ml)

[^{14}C]Leucine

nuclease treated lysate	35µl
H_2O	6µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus leucine)	1µl
RNA substrate in H_2O (see Note 2)	2µl
[^{14}C]leucine (300mCi/mmole) at 50µCi/ml	5µl
	50µl

(Final [^{14}C]leucine concentration = 5µCi/ml)

[^{35}S]Cysteine

nuclease treated lysate	35µl
H_2O	6µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus cysteine)	1µl
RNA substrate in H_2O (see Note 2)	2µl
[^{35}S]cysteine (1,200Ci/mmole) at 10mCi/ml	5µl
	50µl

(Final [^{35}S]cysteine concentration = 1mCi/ml)

[^{35}S]Methionine and [^{3}H]Leucine

nuclease treated lysate	35µl
H_2O	5µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus methionine and leucine)	1µl
RNA substrate in H_2O (see Note 2)	2µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	3µl
[^{3}H]leucine (100-200Ci/mmole) at 5mCi/ml	3µl
	50µl

(Final [^{35}S]methionine concentration = 0.6mCi/ml, [^{3}H]leucine concentration = 0.3mCi/ml)

BMV Positive Control RNA

Brome Mosaic Virus (BMV) RNA (8) is provided as a positive control with each order. For positive control reactions, add 1µg of BMV RNA (at 0.5µg/µl) to the 50µl reaction. In the translation of BMV RNA, four viral proteins are synthesized: 109,000 daltons, 94,000 daltons, 35,000 daltons, and the 20,000 dalton coat protein. A fifth product at 15,000 daltons may also be observed which is a translation product related to the coat protein. BMV RNA is capped but not polyadenylated.

Notes:

1. Hemin is included in the reticulocyte lysate because it is a suppressor of an inhibitor of the initiation factor eIF2a. In the absence of hemin, protein synthesis in reticulocyte lysates ceases after a short period of incubation (9).

2. An unfractionated cytoplasmic RNA preparation is 90-95% rRNA and, as a result, translates poorly. Usually such preparations yield no better than 20-30% of the maximum incorporation attainable, and concentrations of 100-200µg/ml (final conc.) of RNA are needed to stimulate translation. In contrast, viral RNAs and poly(A)+ mRNAs (including mRNA transcribed *in vitro*) can be used at much lower concentrations (5-80µg/ml final). A procedure for rapid isolation and poly(A)+ selection of mRNA is provided on pg. 130.

3. Average preparations of mRNA give a stimulation over background of about 10- to 20-fold. The BMV RNA provided stimulates translation more than 50-fold over background. If the translation efficiency of sample RNA is low, the following suggestions may be utilized.

   a. The optimal RNA concentration for translation should be determined prior to performing definitive experiments. In determining the optimal concentration, serially dilute your RNA template <u>first</u> and then add the same <u>volume</u> of RNA to each reaction to ensure that other variables are kept constant.

   b. To determine if inhibitors are present in your mRNA preparation, mix this RNA with BMV RNA and determine if the translation of viral RNA is inhibited relative to a control translation containing only BMV RNA. Oxidized thiols,

II. Rabbit Reticulocyte Lysate, Nuclease Treated

(continued)

low concentrations of double-stranded RNA, and polysaccharides are typical inhibitors of translation in rabbit reticulocyte lysate (9).

c. Optimum potassium concentration varies from 80-120mM depending on the mRNA used. Additional potassium can be added if the initial translation results are poor. Similarly, specific mRNAs may also require altered magnesium concentrations. A range between 0.5-2.5mM is generally sufficient for the majority of mRNAs utilized. See Tables 1 and 2 (below) for the concentrations of key components present.

d. Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease and result in degradation of the RNA template.

e. The addition of spermidine at approximately 0.4mM has been noted to increase translation efficiency in certain cases (10).

f. Residual ethanol should be removed from mRNA preparations and labeled amino acids before they are added to the translation reaction.

Table 1. Final Concentrations of Rabbit Reticulocyte Lysate Components in a 50µl Translation Reaction.

Creatine phosphate	10mM
Creatine phosphokinase	50µg/ml
DTT	2mM
Calf liver tRNA	50µg/ml
Potassium acetate	79mM
Magnesium acetate	500µM
Hemin	20µM

4. Use capped plastic vials. This avoids changes in reaction volume which may affect the concentration of important components.

5. The addition of RNasin ribonuclease inhibitor to the translation reaction is recommended, but not required. RNasin ribonuclease inhibitor acts to inhibit degradation of sample mRNAs by contaminating RNase.

6. Except for the actual translation incubation, all handling of lysate components should be done at 4°C. Any unused lysate should be rapidly refrozen as soon as possible after thawing to minimize loss of translational activity.

7. Each batch of lysate contains about 100mg/ml of endogenous protein.

8. ^{35}S-labeled amino acids are easily oxidized to sulphoxides. This problem can be minimized by storage in aliquots at –70°C in the presence of 1mM dithiothreitol.

Table 2. Approximate Endogenous Amino Acid Pools (µM)*.

Ala	157	Leu	5
Asn	51	Lys	51
Asp	1093	Met	5
Arg	41	Phe	4
Cys	2	Pro	87
Gln	200	Ser	93
Glu	260	Thr	59
Gly	1050	Trp	1
His	14	Tyr	3
Ile	9	Val	30

*These amino acid concentrations should be used only as estimates. These values are not determined for individual lots of reticulocyte lysate.

Translation *in vitro*

III. Rabbit Reticulocyte Lysate, Untreated

A. Description

Untreated rabbit reticulocyte lysate contains the cellular components necessary for protein synthesis (tRNA; rRNA; amino acids; initiation, elongation, and termination factors), but has not been treated with micrococcal nuclease. Untreated lysate is primarily used for the isolation of these components and as an abundant source of endogenous globin mRNA. Untreated lysate is prepared from New Zealand White rabbits injected with phenylhydrazine using a standard protocol (4) which ensures consistent reticulocyte production. The reticulocytes are purified to remove contaminating cells which could otherwise alter the translational properties of the final extract. The packed cells are then lysed by the addition of water. Untreated rabbit reticulocyte lysate is divided into 1ml aliquots and quick frozen.

Untreated reticulocyte lysate, when supplemented only with hemin (20µM final conc.), synthesizes protein at approximately the same rate as intact cells for up to 60 minutes. The primary disadvantage of such a system for translation *in vitro* is that the added mRNA is translated in competition with the endogenous globin mRNA, making quantitation of the activity of the exogenous RNA very difficult. To overcome this problem, the lysate may be treated with micrococcal nuclease to destroy endogenous mRNA (9). In the presence of calcium as a cofactor, nuclease treatment is very effective in eliminating globin mRNA using incubation times as short as 20 minutes. EGTA is then added to chelate the calcium and thereby inactivate the nuclease. The lysate may be further optimized by addition of the components listed in Table 1. Standard stock solutions and their preparation are as follows:

Composition of Solutions

Hemin (1mM):

Add 65mg hemin hydrochloride to 2.5ml of 1M KOH. Add 1ml of Tris-HCl, pH 7.9, and 89ml of ethylene glycol. Adjust to pH 6.8 with 1N HCl. Add sterile, RNase-free water to a final volume of 100ml. Store in a light-proof bottle at –20°C.

Creatine phosphokinase:

5mg/ml in 20mM Tris-HCl, pH 7.6, 50% glycerol. Store at –20°C.

Creatine phosphate:

0.2M in sterile, RNase-free water. Make up fresh for each use.

DTT:

0.25M solution in sterile, RNase-free water. Store at –20°C.

Potassium acetate:

2.0M solution in RNase-free water. Filter sterilize. Store at –20°C.

Magnesium acetate:

0.1M solution in RNase-free water. Filter sterilize. Store at –20°C.

Calf liver tRNA:

10mg/ml in sterile, RNase-free water. Store at –20°C.

Please see pg. 157 (Nuclease Treated Lysate) for a standard translation protocol.

Promega

IV. Wheat Germ Extract

A. Description

Cell-free extracts of wheat germ support the translation *in vitro* of a wide variety of viral, prokaryotic, and eukaryotic mRNAs into protein (11). Promega's wheat germ extract is prepared by grinding wheat germ in an extraction buffer, followed by centrifugation to remove cell debris. The supernatant is then separated by chromatography from endogenous amino acids and plant pigments that are inhibitory to translation. The extract is also treated with micrococcal nuclease to destroy endogenous mRNA and thus reduce background translation to a minimum. The extract contains the cellular components necessary for protein synthesis: tRNA; rRNA; and initiation, elongation, and termination factors. The extract is further optimized by the addition of the following:

- An energy generating system consisting of phosphocreatine kinase and phosphocreatine.
- Spermidine to stimulate the efficiency of chain elongation and thus overcome premature termination.
- Magnesium acetate at a level recommended for the translation of most mRNA species.

Only the addition of exogenous amino acids (including an appropriate labeled amino acid) and mRNA are necessary to stimulate translation. Potassium acetate is supplied as an individual component so that the translational system may be additionally enhanced for a wide range of mRNAs.

B. Translation Procedure

Materials Required

(See list of reagents and materials needed for reticulocyte lysate translations, pg. 157.)

About 2 hours should be allowed to perform the translation reaction. The SDS gel can conveniently be set up and cast (1 hour) during this time. Allow 2 hours to perform TCA precipitation and preparation of filters for scintillation counting. Two hours should also be allowed to load and run the SDS gel, with an additional 2 hours for the optional staining and destaining procedures.

Protocol

1. Remove the reagents from storage and allow them to thaw slowly on ice.
2. Heat the template mRNA at 67°C for 10 minutes and immediately cool on ice. This increases the efficiency of translation, especially of GC-rich mRNA, by destroying local regions of secondary structure.
3. Assemble the reaction components appropriate to the label being used (see Standard Reactions, below) in a 0.5ml microcentrifuge tube. Gently mix the extract with the pipettor upon addition of each component. If necessary, spin briefly in a microcentrifuge to return the sample to the bottom of the tube. (**Note:** We recommend including a control reaction containing no added mRNA. This allows measurement of any background incorporation of labeled amino acids.)
4. Incubate the wheat germ translation reactions at 25°C for 60 minutes.
5. Analyze the results of translation. Procedures are provided for incorporation assays (pg. 168) and gel analysis of translation products (pg. 170).

Examples of Standard Reactions:

[^{35}S]Methionine

wheat germ extract	25µl
RNasin® ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus methionine)	4µl
1M potassium acetate (see Note 2.b, pg. 162)	0-7µl
RNA substrate in H_2O (see Note 1, pg. 162)	2µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	2.5µl
H_2O	to final volume 50µl

(Final [^{35}S]methionine concentration = 0.5mCi/ml)

Translation *in vitro*

IV. Wheat Germ Extract

(continued)

[^{3}H]Leucine

wheat germ extract	25µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus leucine)	4µl
1M potassium acetate (see Note 2.b)	0-7µl
RNA substrate in H_2O (see Note 1)	2µl
[^{3}H]leucine (100-200Ci/mmole) at 5mCi/ml	3µl
H_2O	to final volume 50µl

(Final [^{3}H]leucine concentration = 0.3mCi/ml)

[^{14}C]Leucine

wheat germ extract	25µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus leucine)	4µl
1M potassium acetate (see Note 2.b)	0-7µl
RNA substrate in H_2O (see Note 1)	2µl
[^{14}C]leucine (300mCi/mmole) at 50µCi/ml	3µl
H_2O	to final volume 50µl

(Final [^{14}C]leucine concentration = 3µCi/ml)

[^{35}S]Cysteine

wheat germ extract	25µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus cysteine)	4µl
1M potassium acetate (see Note 2.b)	0-7µl
RNA substrate in H_2O (see Note 1)	2µl
[^{35}S]cysteine (1,200Ci/mmole) at 10mCi/ml	2.5µl
H_2O	to final volume 50µl

(Final [^{35}S]cysteine concentration = 0.5mCi/ml)

[^{35}S]Methionine and [^{3}H]Leucine

wheat germ extract	25µl
RNasin ribonuclease inhibitor (at 40u/µl)	1µl
1mM amino acid mixture (minus methionine and leucine)	4µl
1M potassium acetate (see Note 2.b)	0-7µl
RNA substrate in H_2O (see Note 1)	2µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	1.5µl
[^{3}H]leucine (100-200Ci/mmole) at 5mCi/ml	1.5µl
H_2O	to final volume 50µl

(Final [^{35}S]methionine concentration = 0.3mCi/ml, [^{3}H]leucine; concentration = 0.15mCi/ml)

Technical Services: 800-356-9526

BMV Positive Control RNA

Brome Mosaic Virus (BMV) RNA (7) is provided as a positive control with each order. For positive control translations, add 1µg of BMV RNA (at 0.5µg/µl) to the 50µl reaction. In the translation of BMV RNA, four viral proteins are synthesized: 109,000 daltons, 94,000 daltons, 35,000 daltons, and the 20,000 dalton coat protein. A fifth product at 15,000 daltons may also be observed which is a translation product related to the coat protein. BMV RNA is capped but not polyadenylated.

Notes:

1. An unfractionated cytoplasmic RNA preparation is 90-95% rRNA and, as a result, translates poorly. Usually such preparations yield no better than 20-30% of the maximum incorporation attainable, and concentrations of 100-200µg/ml (final conc.) of RNA are needed to stimulate translation. In contrast, viral RNAs and poly(A)+ mRNAs (including mRNA transcribed *in vitro*) can be used at much lower concentrations (5-80µg/ml final). A procedure for rapid isolation and poly(A)+ selection of mRNA is provided on pg. 130.

2. Average preparations of mRNA give a stimulation over background of about 10- to 20- fold. The BMV RNA provided stimulates translation more than 50-fold over background. If the translation efficiency of RNA is low, the following suggestions may be utilized.

   a. The optimal RNA concentration for translation should be determined prior to performing definitive experiments. In determining the optimal concentration, serially dilute your RNA template <u>first</u> and then add the same <u>volume</u> of RNA to each reaction to ensure that other variables are kept constant.

   b. Optimum potassium concentration varies from 50-200mM, depending on the mRNA used. The optimal potassium concentration for translation of BMV RNA is 135mM. If this concentration of potassium results in poor translation of your sample mRNA, potassium levels should be reduced or increased to an optimum concentration. Certain mRNAs may also require altered magnesium concentrations. The optimum magnesium concentration for the majority of mRNAs utilized is expected to fall in the range of 2-5mM. See Table 3 for the concentrations of key exogenous components of wheat germ extract.

IV. Wheat Germ Extract

(continued)

c. Avoid adding calcium to the translation reaction. Calcium may reactivate the micrococcal nuclease and result in degradation of the RNA template.

d. Residual ethanol should be removed from mRNA preparations and labeled amino acids before they are added to the translation reaction.

3. The addition of RNasin® ribonuclease inhibitor to the translation reaction is recommended, but not required. RNasin ribonuclease inhibitor acts to inhibit degradation of sample mRNAs by contaminating RNase.

4. Each batch of wheat germ extract contains 30-50mg/ml of endogenous protein.

5. Except for the actual translation incubation, all handling of wheat germ extract components should be done at 4°C. Any unused extract should be rapidly refrozen as soon as possible after thawing to minimize loss of translational activity.

6. ^{35}S-labeled amino acids are easily oxidized to sulphoxides. This problem can be minimized by storage in aliquots at –70°C in the presence of 1mM dithiothreitol.

Table 3. Final Concentrations of Wheat Germ Extract Components in a 50µl Translation Reaction.

Creatine phosphate	10mM
Creatine phosphokinase	50µg/ml
DTT	5mM
Calf liver tRNA	50µg/ml
Magnesium acetate	2.5mM
Potassium acetate	60mM*
Spermidine	0.5mM
ATP	1.2mM
GTP	0.1mM
HEPES	12mM

*Additional potassium acetate may need to be added to optimize translation for each sample RNA. See Note 2.c.

Translation *in vitro*

V. Cotranslational Processing Using Canine Pancreatic Microsomal Membranes

A. Description

Microsomal vesicles are used to study cotranslational and initial post-translational processing of proteins. Processing events such as signal peptide cleavage, membrane insertion, translocation, and core glycosylation can be examined by the translation of the appropriate mRNA *in vitro* in the presence of these microsomal membranes. To assure consistent performance with minimal translational inhibition and background, microsomes have been isolated free from contaminating membrane fractions and stripped of endogenous membrane bound ribosomes and mRNA. Membrane preparations are assayed for both signal peptidase and core glycosylation activities using two different control mRNAs (Figure 1). The two control mRNAs supplied with this system are the precursor for β-lactamase (or ampicillin resistance gene product) from *E. coli* and the precursor for α-mating factor (or α-factor gene product) from *S. cerevisiae*.

Unit Definition: The activity of the membranes is defined in equivalents (7). One equivalent is that amount of membranes required to cleave the signal sequence of preprolactin from 50% of the translation products, as measured by a shift in mobility on SDS-polyacrylamide gels. Typically, 1.8µl of Promega's membranes per 25µl of translation mix will process 90% of the signal sequence positive control and 75-90% of the core glycosylation positive control.

B. Cotranslational Processing Procedure

To test the processing efficiencies of the microsomal membranes, the following procedure can be performed with the positive control mRNAs supplied. This protocol is used at Promega to assay the efficiency of protein processing by microsomal membranes. While these reaction conditions will be suitable for most applications, the efficiency of processing using alternate translation systems, mRNAs, or membranes may vary. Thus, reaction parameters may have to be altered to suit individual requirements. In general, increasing the amount of membranes in the reaction increases the proportion of polypeptides translocated into vesicles but reduces the total number of polypeptides synthesized.

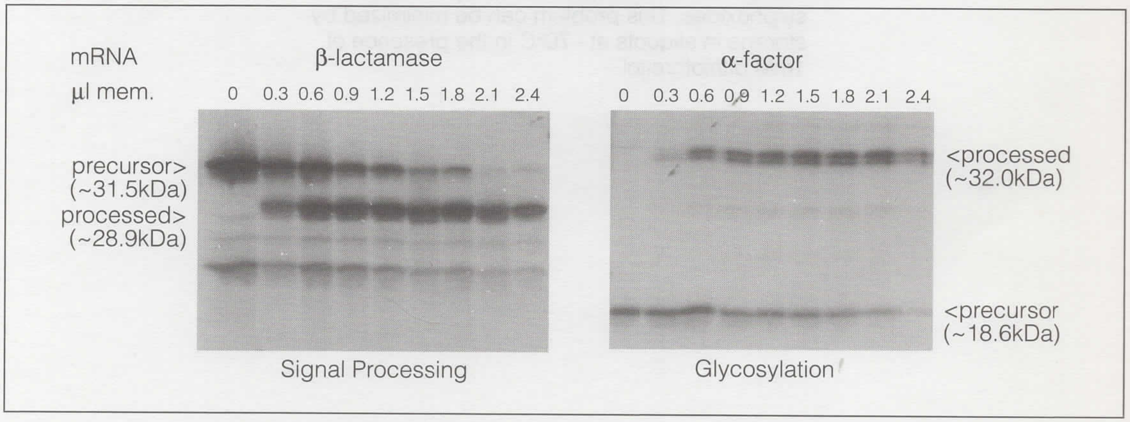


Figure 1. Processing and glycosylation activity of canine pancreatic microsomal membranes. The positive control mRNAs (0.5µg each of *E. coli* β-lactamase and *S. cerevisiae* α-factor) were translated using Promega's rabbit reticulocyte lysate in a 25µl reaction for 60 minutes in the presence of the indicated amounts of microsomal membranes. Aliquots (3µl) were then analyzed by gel electrophoresis and autoradiography of the ^{35}S-labeled proteins.

V. Cotranslational Processing Using Canine Pancreatic Microsomal Membranes

(continued)

Materials

(See list of reagents and materials needed for reticulocyte lysate translations, pg. 157.)

About 2 hours should be allowed to perform the translation reaction. The SDS gel can conveniently be set up and cast during this time. Allow 2 hours to perform TCA precipitation and preparation of filters for scintillation counting. Two hours should also be allowed to load and run the SDS gel, with an additional 2 hours for the optional staining and destaining procedures.

Protocol

1. Remove the reagents from the freezer and allow them to thaw on ice.
2. Mix the following components on ice, in the order given, in a sterile microcentrifuge tube:

nuclease treated rabbit reticulocyte lysate	17.5µl
1mM amino acid mixture (minus methionine)	0.5µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	2.0µl
H_2O	2.2µl
microsomal membranes	1.8µl
RNA substrate in H_2O (pre β-lactamase and α-factor mRNA at 0.5µg/µl)	1.0µl
	25.0µl

3. Incubate at 30°C for 60 minutes.
4. Analyze the results of translation and processing. Procedures are provided for incorporation assays (pg. 168) and gel analysis of translation products (pg. 170). For information on assays of cotranslational processing, see Note 5, below.

Notes:

1. 1.8µl of microsomal membranes per 25µl of translation mix will process 90% of pre-β-lactamase to β-lactamase. The same amount of membranes will process 75-90% of α-factor to core glycosylated forms of α-factor.
2. When analyzed by SDS gel electrophoresis, the precursor for β-lactamase migrates at 31.5kDa and the processed β-lactamase at 28.9kDa. The precursor for the α-factor migrates at 18.6kDa, and the core-glycosylated α-factor migrates at 32.0kDa. See Figure 1 (pg. 164) for an example of SDS gel analysis of these translation products.
3. Depending on the mRNA under examination, translation efficiency can be expected to drop between 10-50% in the presence of microsomal membranes.
4. Storage buffer for the microsomal membranes consists of 50mM triethanolamine, 2mM DTT, and 250mM sucrose.
5. In some cases, it is difficult to determine by gel analysis alone if efficient processing or glycosylation has occurred. Two additional assays for detecting cotranslational processing events are described below.

A general assay for cotranslational processing makes use of the protection afforded the translocated protein domain by the lipid bilayer of the microsomal membrane. In this assay, protein domains are judged to be translocated if they are observed to be protected from exogenously added protease. To confirm that protection is due to the lipid bilayer, addition of 0.1% non-ionic detergent (such as Triton X-100 or Nikkol) solubilizes the membrane and restores susceptibility to protease. Many proteases have proven useful for monitoring translocation in this fashion including proteinase K and trypsin (final concentration 0.1mg/ml) (12).

An alternative procedure utilizes endoglycosidase H to determine the extent of glycosylation of translation products (12). In cell-free systems, N-linked glycosylation occurs only within intact microsomes. Endoglycosidase H cleaves the internal N-acetylglucosamine residues of high mannose carbohydrates, resulting in a shift in apparent molecular weight on SDS gels to a position very close to that of the non-glycosylated species. The reaction conditions (0.1% SDS, 0.1M sodium citrate, pH 5.5, incubation at 37°C for 12 hours) are not compatible with those required to maintain membrane integrity. For this reason, translocated polypeptides are not "protected" from digestion with endoglycosidase H.

VI. *E. coli* S30 Coupled Transcription Translation System

A. Description

The *E. coli* S30 coupled transcription translation systems simplify the transcription of DNA sequences cloned in plasmid vectors, providing a powerful tool for identifying and characterizing polypeptides. The investigator need only supply the cloned DNA containing the appropriate prokaryotic promoter and ribosome binding sites.

Two types of S30 systems are available. The standard systems allow for the expression of cloned DNA fragments present in supercoiled plasmid vectors under control of an *E. coli* promoter. Linear DNA templates are degraded in these systems.

The second type of S30 system is generated from an *E. coli* strain that allows either plasmid or linear DNA to be transcribed and subsequently translated. This is possible due to a *rec*D mutation that inactivates exonuclease V.

Promega's S30 extracts are prepared from *E. coli* strains deficient in *omp*T endoproteinase and *lon* protease activity. This results in a greater stability of expressed proteins which would otherwise be degraded by proteases if expressed *in vivo* (13,14). The S30 *in vitro* system also allows higher level expression of proteins which are normally expressed at low levels *in vivo* due to the action of host-encoded repressors (15). This system is ideal for confirming results obtained *in vivo* (e.g., with mini or maxi cells), and simplifies the analysis of factors controlling gene expression or promoter efficiency (16).

Promega's S30 extracts, prepared by modifications of the method described by Zubay (2,3), are available in several systems. In each system, one premix contains all 20 amino acids and can synthesize unlabeled proteins, while the other premix lacks a particular amino acid(s) and is used in protein labeling experiments. Both premixes are optimized for each lot of S30 extract and contain all other required components, including NTPs, tRNAs, a PEP energy regenerating system and appropriate salts. Also included is a control DNA containing the *lac*Z gene which is transcribed from the *lac* promoter to test the system (Section C, pg. 167). Expression of functional β-galactosidase enzyme can be easily assayed colorimetrically using the ONPG and Na_2CO_3 supplied (17), or the total TCA precipitable radioactivity can be determined (pg. 168).

The most common application of the S30 extract is for the synthesis of small amounts of radiolabeled protein. The synthesis of a protein of the correct size is a useful means of verifying the gene product of a particular DNA sequence. Proteins expressed in the S30 extract also may be used for a variety of functional studies of transcription and translation (18). Some additional applications of the S30 extract include synthesis of small amounts of radiolabeled protein for use as a tracer in protein purification (18) and incorporation of unnatural amino acids into proteins for structural studies (19). The S30 extract for linear DNA templates is particularly useful for epitope mapping studies. In these experiments, the gene of interest is truncated by restriction digestion or by PCR using appropriate primers and the expressed polypeptide is monitored for the loss of specific epitopes (20).

B. Template Requirements

Expression of cloned DNA fragments in the S30 system requires that the gene be under the control of a good *E. coli* promoter. Examples of such good promoters include the lambda P_I, lambda P_L, *tac*, *trc* and *lac*UV5. The DNA template added must be highly purified. DNA templates to be used with the S30 systems should be purified by CsCl gradient centrifugation or by electrophoresis. Care should be taken to avoid adding excessive salts or glycerol when adding the DNA template. The activity of the S30 system may be inhibited by NaCl ≥50mM, glycerol ≥1%, or by very small amounts of Mg^{2+} or potassium salts. Thus, the DNA template should be precipitated with sodium acetate rather than ammonium acetate. The total amount of protein that may be synthesized with the S30 system varies with the template used and the conditions of use, and is typically 50-250ng per reaction.

VI. *E. coli* S30 Coupled Transcription Translation System

(continued)

C. Coupled Transcription Translation Procedure

Materials to be Supplied by the User

(See list of reagents and materials needed for reticulocyte lysate translations, pg. 157.)

1. Set up the following reaction with sample DNA:

DNA substrate + H_2O (total of 2µg DNA, see Note 1)	14µl
premix minus methionine (mix gently prior to use)	20µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml (see Note 2)	1µl
S30 extract (mix gently prior to use)	15µl
	50µl

Gently vortex, then centrifuge briefly.

2. Set up the following reaction with the control DNA provided:

H_2O	10µl
control DNA (total of 2µg)	4µl
premix minus methionine (mix gently prior to use)	20µl
[^{35}S]methionine (1,200Ci/mmole) at 10mCi/ml	1µl
S30 extract (mix gently prior to use)	15µl
	50µl

Gently vortex, then centrifuge briefly.

Note: To generate unlabeled β-galactosidase for colorimetric assays (Section D, below), use the complete amino acid mixture provided rather than the mixture lacking methionine.

3. Incubate the reaction(s) at 37°C for 30-60 minutes.

4. Analyze the results of the reaction. Procedures are provided for incorporation assays (pg. 168) and gel analysis of proteins synthesized (pg. 170).

Notes:

1. The optimal amount of DNA added to the extract can vary. We recommend 2µg of the provided positive control for routine analysis.

2. For optimal results [^{35}S]methionine should be stored in aliquots at –70°C, used once and discarded.

3. The positive control DNA provided may be used to synthesize the β-galactosidase protein. Unlabeled, this active β-galactosidase can be used in a colorimetric assay to monitor the efficiency of the S30 reaction. As a labeled protein, it can be visualized as a band migrating at 114kDa. The precursor form of β-lactamase may also be noted as a faint band migrating at 31.5kDa.

4. Water purity is extremely important. If poor efficiencies are noted, use an alternative source of water in the S30 reaction.

D. Synthesis and Colorimetric Assay of β-Galactosidase Control

Reagents Needed

- ONPG solution (supplied)
- 1M Na_2CO_3 (supplied)

1. Set up the following reaction with the pGEMβGAL control DNA provided.

H_2O	11µl
DNA (2µg)	4µl
complete premix (supplied)	20µl
S30 extract	15µl
	50µl

Incubate the reaction at 37°C for 60 minutes.

2. Stop the reaction by placing it in an ice bath for 5 minutes.

3. Add 450µl of ONPG solution (supplied) (pg. 168). Mix gently.

4. Incubate at 30°C until a yellow color is noted (usually 5 minutes).

5. Note the time and then add 250µl of 1M Na_2CO_3.

6. Place the reaction on ice for 10 minutes.

Translation *in vitro*

VI. *E. coli* S30 Coupled Transcription Translation System

(continued)

7. Centrifuge for 5 minutes at 12,000 x g.
8. Read the OD_{420}.
9. Calculate the units of β-galactosidase activity as shown below.

$$\text{Units} = \frac{1{,}000 \times (OD_{420} - OD_{420} \text{ of no DNA control})}{\text{time (minutes)}}$$

In this equation, time refers to the assay incubation time used in Step 4.

Composition of Solutions

ONPG Solution:

60mM Na_2HPO_4
40mM NaH_2PO_4
10mM KCl
1mM $MgSO_4$
50mM β-mercaptoethanol

Adjust to pH 7.0 and then add ONPG to 3mM.

VII. TCA Protein Precipitation Assay for Amino Acid Incorporation

Reagents Needed

- 1N NaOH/2% H_2O_2 (for reticulocyte lysate)
- 1N NaOH (for wheat germ or S30)
- 25% TCA/2% casamino acids
- 5% TCA

A. TCA Precipitation Procedure

The following protocol is used for the determination of labeled amino acid incorporation into protein during a typical translation reaction.

1. Vortex the translation reaction gently prior to removing an aliquot to be analyzed for incorporation. Use a 2µl aliquot for reticulocyte lysate and a 5µl aliquot for S30 or wheat germ extract assays.

   To determine background counts, remove 2 or 5µl from a reaction containing no mRNA and proceed as described in Steps 2-6.

2. Dilute the aliquot to a final volume of 250µl with 1N NaOH/2% H_2O_2 solution (for reticulocyte lysate samples) or 1N NaOH (for wheat germ or S30 samples) in a 1.5ml microcentrifuge tube, mix and incubate at 37°C for 10 minutes. The NaOH hydrolyzes aminoacyl tRNAs and thus prevents labeled tRNA from being included in the incorporation calculation. Hydrogen peroxide removes the red color from the reticulocyte lysate reaction, thus preventing any quenching during scintillation counting.

3. At the end of the 10 minute incubation, add 1.0ml of ice-cold 25% TCA/2% casamino acids to precipitate the translation products. (The casamino acids act as carriers.) Incubate on ice for 30 minutes.

4. Collect the precipitate by filtering under vacuum on Whatman GF/A glass fiber filters. Wet the filter with a small amount of ice-cold 5% TCA. Filter the sample and rinse the filter 3 times with 3ml of ice-cold 5% TCA. Rinse once with 1-3ml acetone. Allow the filter to dry completely at room temperature or dry it at about 75°C for 10 minutes.

5. For determination of ^{35}S or ^{14}C incorporation, put the filter in 1-3ml of an appropriate scintillation mixture, invert to mix and count. For ^{3}H, add the same amount of scintillation mixture, invert, but leave the scintillation vials in the dark for 30 minutes at room temperature prior to counting.

6. To determine total counts present in the translation reaction, spot a 2-5µl aliquot of the reaction mix directly onto a filter and allow it to dry. Count in a liquid scintillation counter as in Step 5. **Note:** Pigments present in the translation reactions may lead to quenching of this sample, and must be corrected for.

VII. TCA Protein Precipitation Assay for Amino Acid Incorporation

(continued)

B. Sample Calculation to Determine the Efficiency of Translation

1. Total radioactivity/µl is first determined (see Step A.7):

$$\frac{\text{amount of radioactivity determined}}{\mu\text{l of reaction filtered}}$$

Example: $\frac{4 \times 10^6 \text{ cpm}}{2\mu\text{l}} = 2 \times 10^6 \text{ cpm}/\mu\text{l}$

2. Total radioactivity in the translation reaction (typical volume is 50µl):

$$\frac{\text{amount of radioactivity} \times \text{total reaction volume}}{\mu\text{l of reaction filtered}}$$

Example: $2 \times 10^6 \text{ cpm}/\mu\text{l} \times 50\mu\text{l} = 1 \times 10^8 \text{ cpm}$

3. Number of TCA precipitable counts (cpm incorporated into protein) of an aliquot of the translation reaction (see step A.5):

$$\frac{\text{number of counts incorporated into protein}}{\mu\text{l of reaction TCA precipitated}}$$

Example: $\frac{1 \times 10^6}{2\mu\text{l}} = 5 \times 10^5 \text{ cpm}/\mu\text{l}$

4. Total number of counts incorporated into protein in a standard reaction (volume = 50µl):

cpm per microliter x total volume

Example: $5 \times 10^5 \text{cpm}/\mu\text{l} \times 50\mu\text{l} = 2.5 \times 10^7 \text{ cpm}$

5. Percent incorporation:

$$\frac{\text{total radioactivity incorporated into protein}}{\text{total radioactivity in the reaction}} \times 100$$

Example: $\frac{2.5 \times 10^7 \text{cpm}}{1 \times 10^8 \text{cpm}} \times 100 = 25\% \text{ incorp.}$

VIII. SDS Gel Analysis of Translation Products

The most widely applicable and versatile method for analysis of cell-free translation products synthesized from mixtures of RNAs is polyacrylamide slab gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS) and a discontinuous buffer system. The separating gel at a concentration of 15% acrylamide gives good separation of peptide mixtures between 20,000 and 100,000 molecular weight (MW), with peptides between 55,000 and 60,000 MW migrating halfway down the length of the gel.

An alternative to standard SDS gel electrophoresis is Promega's recently developed gel system, in which proteins are stained as they migrate through the gel. This system is described on pg. 245.

Reagents Needed

Instructions for preparing these solutions are provided on pg. 172.

- SDS sample buffer
- 0.1% SDS
- 30% acrylamide solution
- separating gel 4X buffer
- stacking gel 4X buffer
- reservoir 10X buffer
- staining solution
- destaining solution

A. Sample Preparation

1. **For reticulocyte lysate or wheat germ samples:**

   Once the reticulocyte lysate or wheat germ extract translation is complete (or at any desired time point), remove a 5µl aliquot, add it to 20µl of loading 2X buffer (pg. 172) and heat the sample at 100°C for 2-5 minutes. A small aliquot of the sample may be loaded on an SDS gel or the sample may be stored at –20°C. Because a large fraction of the label is incorporated into protein with these systems, it is not necessary to separate labeled polypeptides from free amino acids by acetone precipitation. Proceed to Section B.

   For S30 extract samples:

   Once the S30 extract reaction is complete (or at any desired time point), remove a 5µl aliquot, add it to 20µl of acetone in a microcentrifuge tube and place it on ice for 15 minutes. The unused portion of the reaction may be stored at –20°C.

2. Centrifuge the acetone-precipitated S30 sample at 12,000 x g for 5 minutes.

3. Remove the supernatant and dry the pellet for 15 minutes under vacuum.

4. When the pellet is dry, add 20µl of SDS sample buffer and heat at 100°C for 2-5 minutes. A small aliquot of the sample may be loaded on an SDS gel or the sample may be stored at –20°C.

B. Preparation and Running of SDS-Polyacrylamide Gels

Formulations for preparing separating and stacking minigels are provided on pg 171.

1. Pour the separating gel mix into the assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. Gently overlay the gel mix with 0.1% SDS.

2. After polymerization (10-15 minutes), remove the overlay and rinse the surface of the separating gel first with water to remove any unpolymerized acrylamide and then with a small volume of stacking gel mix.

3. Fill the remaining space with stacking gel mix and insert the comb immediately.

4. After the stacking gel has polymerized (30-45 minutes), remove the comb and rinse the wells to remove unpolymerized acrylamide.

5. Load a small aliquot of the heated sample on the gel. Load 5µl (for reticulocyte lysate samples) or 10µl (for S30 or wheat germ extract samples).

6. Typically, electrophoresis is carried out at a constant current of 15mA in the stacking gel and 30mA in the separating gel. Electrophoresis is usually performed until the bromophenol blue dye front has run off the bottom of the gel.

   Note: Gel banding patterns may be improved by loading unlabeled samples of reticulocyte lysate, wheat germ extract or S30 extract in the lanes adjacent to the radioactive sample lanes.

VIII. SDS Gel Analysis of Translation Products

(continued)

C. Staining of SDS Gels

After electrophoresis, protein bands in the gel may be visualized by staining with Coomassie blue dye. Coomassie staining, however, is usually not sensitive enough to detect translation products, and thus need not be performed before analyzing gel results by fluorography. The staining and destaining steps do, however, help to wash out unincorporated labeled amino acids at the gel dye front.

1. Stain with gentle agitation until the dye has penetrated the gel (15-30 minutes).

2. Dye that is not bound to protein is removed by transferring the gel to destaining solution (pg. 172). Add a wad of laboratory tissues to absorb excess stain and gently agitate in this solution until bands are clearly visible (changing the solution may be required).

3. Cut a sheet of Whatman® 3MM paper a little larger than the gel itself. Place this under the gel once destaining has been completed. Transfer the gel to a vacuum gel drier. Place plastic wrap over the gel and dry for 1 hour at 60°C followed by 1 hour at room temperature.

   Alternatively, the gel may be air dried using Promega's gel drying frames. Soak the gel in 10% glycerol for 30 minutes to prevent the gel from cracking during drying. Place the gel between two sheets of thoroughly moistened cellulose gel drying film and clamp in the frames. Allow the gel to dry overnight.

D. Fluorography

Following electrophoresis, labeled protein bands in gels may be visualized by autoradiography or fluorography. Fluorography dramatically increases the sensitivity of detection of ^{35}S, ^{14}C and ^{3}H-labeled proteins, and is recommended for the analysis of translation *in vitro* products. Autoradiography is sufficiently sensitive to detect ^{35}S-labeled BMV RNA translation products using an 8-16 hour exposure to film (Kodak X-OMAT AR).

The increased detection sensitivity of fluorography is obtained by infusing an organic scintillant into the gel. The scintillant converts the emitted energy of the isotope to visible light and so increases the proportion of energy which may be detected by X-ray film. Commercial reagents are available which can conveniently be used for fluorographic enhancement of signal.

Table 5. Formulation of Stacking Gel (5% Acrylamide).

Component	Volume
H_2O	1.0ml
30% Acrylamide solution (pg. 172)	300µl
Stacking gel 4X buffer (pg. 172)	444µl
10% Ammonium persulfate	28µl
TEMED	5µl

Table 4. Formulation for SDS-Polyacrylamide Separating Gels.
(Recipes are sufficient for the preparation of a slab minigel 0.75mm thick, 10cm x 7cm.)

	Volume for different percentages of acrylamide				
Component	8.5%	10%	12.5%	15%	17.5%
H_2O	3.5ml	3.1ml	2.5ml	1.8ml	1.2ml
30% Acrylamide solution (pg. 172)	2.0ml	2.4ml	3.0ml	3.7ml	4.3ml
Separating gel 4X buffer (pg. 172)	1.9ml	1.9ml	1.9ml	1.9ml	1.9ml
10% Ammonium persulfate	112µl	112µl	112µl	112µl	112µl
TEMED	5µl	5µl	5µl	5µl	5µl

VIII. SDS Gel Analysis of Translation Products

(continued)

Composition of Solutions

Separating gel 4X buffer (per 100ml):

18.17g	Tris base
4ml	10% SDS

Adjust to pH 8.8 with 12N HCl and add H_2O to a final volume of 100ml. Store at room temperature.

30% Acrylamide solution (per 100ml):

30g	acrylamide
0.8g	bisacrylamide

Add H_2O to a final volume of 100ml. Store at 4°C.

Stacking gel 4X buffer (per 100ml):

6.06g	Tris-base
4ml	10% SDS

Adjust to pH 6.8 with 12N HCl and add H_2O to a final volume of 100ml. Store at room temperature.

Loading 2X buffer (per 10ml):

2.0ml	glycerol
2.0ml	10% SDS
0.25mg	bromophenol blue
2.5ml	stacking gel 4X buffer
0.5ml	β-mercaptoethanol

Add H_2O to a final volume of 10ml. Store at room temperature.

Running 10X buffer (per liter):

30g	Tris-base
144g	glycine
100ml	10% SDS

Staining solution (per liter):

250ml	isopropanol
100ml	glacial acetic acid
650ml	H_2O
2.5g	Coomassie brilliant blue R250

Store at room temperature.

Destaining solution (per liter):

70ml	glacial acetic acid
830ml	H_2O

Store at room tempertaure.

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X. Additional Translation *in vitro* Literature Available from Promega

Technical Manuals

Translation *in vitro* Technical Manual

Technical Bulletins

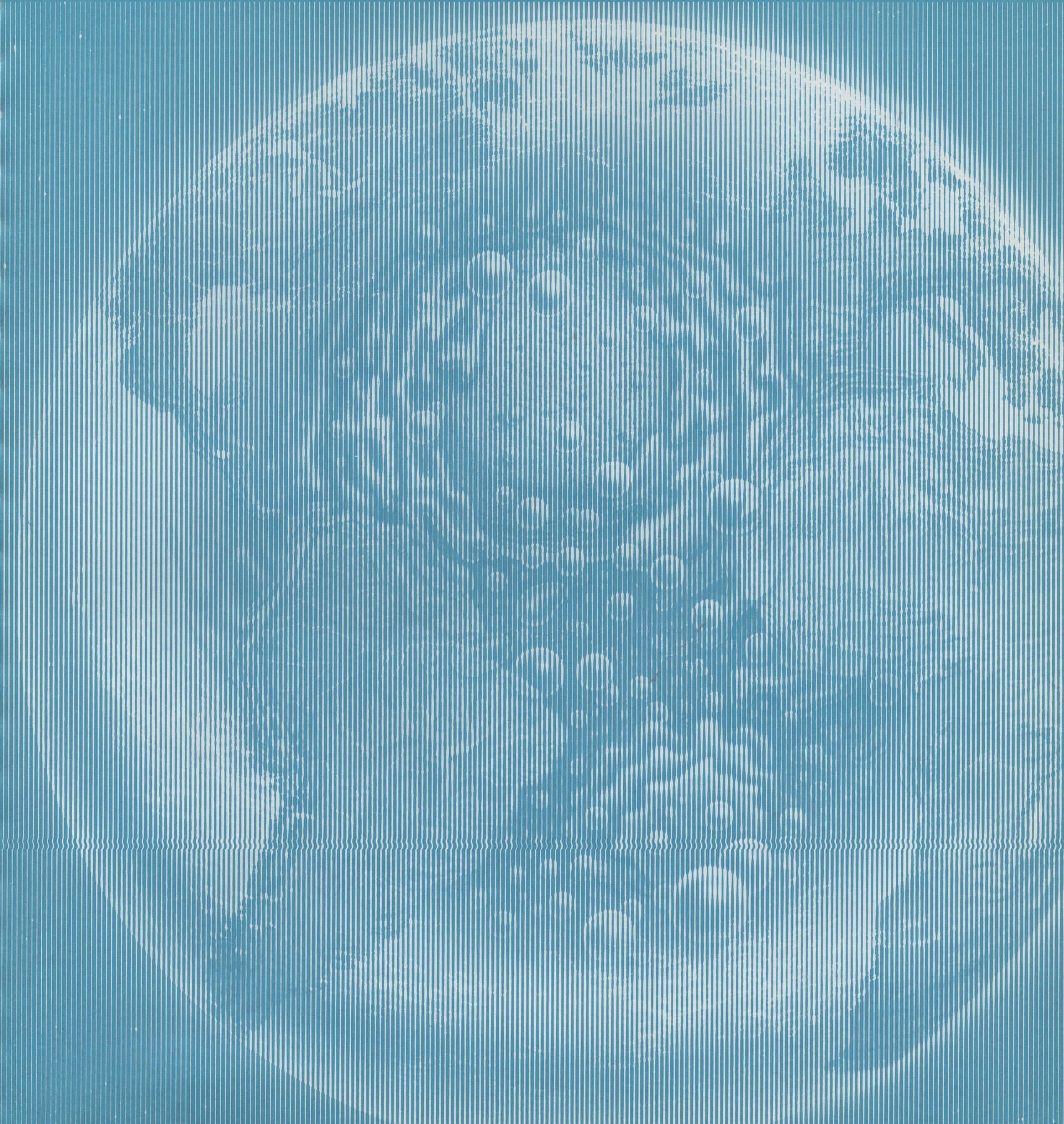
092 *E. coli* S30 Coupled Transcription Translation System

Promega Notes Articles

Issue	Title of Article
9	Wheat Germ Extract
9	Translation in wheat germ extract of *in vitro* synthesized mRNA
11	Assaying protein translocation across the endoplasmic reticulum membrane (microsomes)
17	Microinjection of *in vitro* derived mRNA into amphibian eggs
19	Cell-free translation of plant mRNA in rabbit reticulocyte lysate
23	Improved incorporation with wheat germ extract
26	*E. coli* S30 coupled transcription translation system
26	Post-translational isoprenylation of human lamin B in the reticulocyte lysate *in vitro* translation system

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Genomic Cloning and Mapping

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Genomic Cloning and Mapping

Contents

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I. Lambda Genomic Cloning Vectors: Descriptions, Cloning Strategies and Applications

An optimal strategy for genomic cloning should meet three requirements: 1) a maximum number of recombinants should be obtained with a minimal background of nonrecombinants, 2) the genomic library constructed should be representative of genomic DNA sequences, and 3) the strategy should facilitate restriction mapping of the cloned DNA. Promega's preparations of EMBL3, LambdaGEM®-11, and LambdaGEM®-12 vector arms (see Figure 1) are optimized for the highest recombinant efficiencies and lowest non-recombinant backgrounds possible. When using either Promega's *Bam*H I or *Xho* I half-site lambda arms, non-productive ligations of genomic DNA with the central stuffer fragment are extremely rare (<100pfu/µg arms, or one per 10^5 recombinants). Thus, more recombinant DNA is cloned and larger libraries are generated while fewer filters need to be processed per screening experiment. Because of these low backgrounds, the need for *Spi* selection against parental phage in recombination proficient (*rec*⁺) hosts is eliminated.

Spi (sensitivity to P2 interference) selection (1) is based on the fact that phage replication is inhibited in P2 lysogens when the phage contains functional *red* and *gam* genes (present on the stuffer fragment of the lambda cloning vector). When this stuffer DNA is replaced by the DNA insert in a recombinant phage, replication of the phage can proceed. A serious disadvantage of *Spi* selection is that *rec*BC⁺ hosts must be used, leading to the deletion of potential clones by recombination. This is significant, since recent experiments have demonstrated that 9% of the human and *Drosophila* genomes are not represented in libraries plated on *rec*BC⁺ hosts (2).

Two ultra-low background cloning strategies can be used with Promega's genomic cloning vectors. DNA partially digested with *Mbo* I or *Sau*3A I can be cloned into dephosphorylated *Bam*H I arms (EMBL3 or LambdaGEM-11 vectors). Religation of these arms with the central stuffer fragment has been eliminated by secondary digestion with *Eco*R I and removal of the small *Bam*H I-*Eco*R I fragments. DNA fragments ranging in size from 9-23kb can be cloned in these vectors. However, for library constructions using these *Bam*H I sites, the genomic DNA should be size-fractionated following the partial digestion step to avoid any possibility of cloning two or more genomic fragments into the same site. For this purpose, only fragments greater than 14kb should be selected for ligation with the vector.

The LambdaGEM-11 and -12 vectors can be used for an alternative cloning strategy involving the use of partially filled-in *Xho* I sites in conjunction with partially filled-in *Sau*3A I or *Mbo* I digested genomic DNA (3, see Figure 2). This approach eliminates the need for size-fractionation of genomic fragments, since the partial fill-in reaction prevents insert-to-insert ligation. The only ligation products possible

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I. Lambda Genomic Cloning Vectors: Descriptions, Cloning Strategies and Applications

(continued)

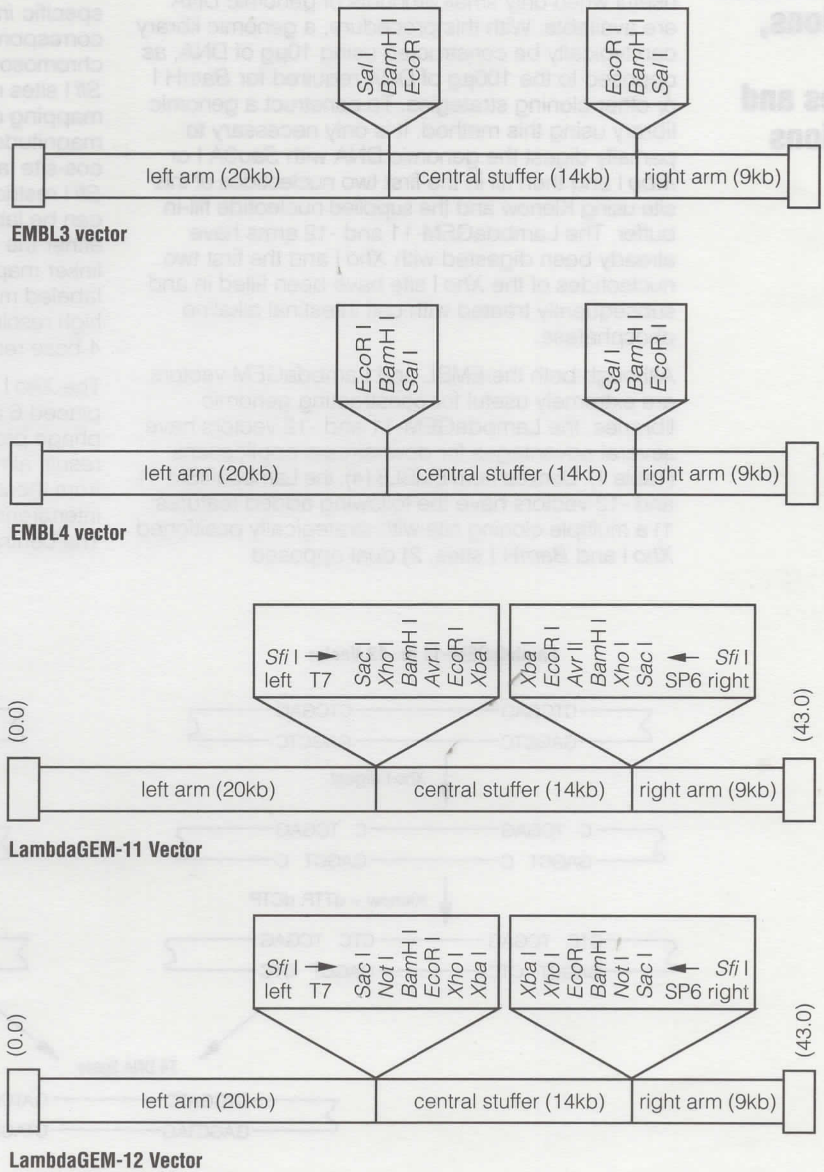

Figure 1. Schematic line maps of EMBL3, EMBL4, LambdaGEM-11 and LambdaGEM-12 cloning vectors. See pgs. 378-381 for additional information.

Genomic Cloning and Mapping

I. Lambda Genomic Cloning Vectors: Descriptions, Cloning Strategies and Applications

(continued)

are single copies of genomic inserts with the appropriate arms. Since any fractionation procedure results in the loss of significant amounts of material, the *Xho* I half-site cloning method is especially useful when only small amounts of genomic DNA are available. With this procedure, a genomic library can typically be constructed using 10µg of DNA, as opposed to the 100µg of DNA required for *Bam*H I or other cloning strategies. To construct a genomic library using this method, it is only necessary to partially digest the genomic DNA with *Sau*3A I or *Mbo* I and then fill in the first two nucleotides of this site using Klenow and the supplied nucleotide fill-in buffer. The LambdaGEM-11 and -12 arms have already been digested with *Xho* I and the first two nucleotides of the *Xho* I site have been filled in and subsequently treated with calf intestinal alkaline phosphatase.

Although both the EMBL and LambdaGEM vectors are extremely useful for constructing genomic libraries, the LambdaGEM-11 and -12 vectors have several advantages for downstream applications (Table 1). Derived from EMBL3 (4), the LambdaGEM-11 and -12 vectors have the following added features: 1) a multiple cloning site with strategically positioned *Xho* I and *Bam*H I sites, 2) dual opposed bacteriophage T7 and SP6 promoters, and 3) asymmetric *Sfi* I restriction sites flanking the cloning/promoter region.

The two bacteriophage promoters allow the simple, specific *in vitro* synthesis of RNA probes corresponding to each end of the insert, useful for chromosome mapping and walking. The flanking *Sfi* I sites make possible high resolution restriction mapping of insert DNA, offering an order of magnitude greater resolution than conventional cos-site lambda mapping allows. From a single *Sfi* I restriction digestion, each end of the insert DNA can be labeled using oligonucleotides specific for either the right or left *Sfi* I site (using Promega's *Sfi* I linker mapping system, see Figure 3, pg. 190). The labeled molecules can then be easily mapped at high resolution using restriction enzymes with 4-base recognition sites.

The *Xho* I and *Bam*H I cloning sites overlap and are placed 6 and 11 bases, respectively, from either phage promoter in the LambdaGEM-11 vector. As a result, almost no vector sequences are transcribed from these promoters, eliminating potential interference in RNA probe hybridization experiments. The central stuffer regions of the LambdaGEM-11

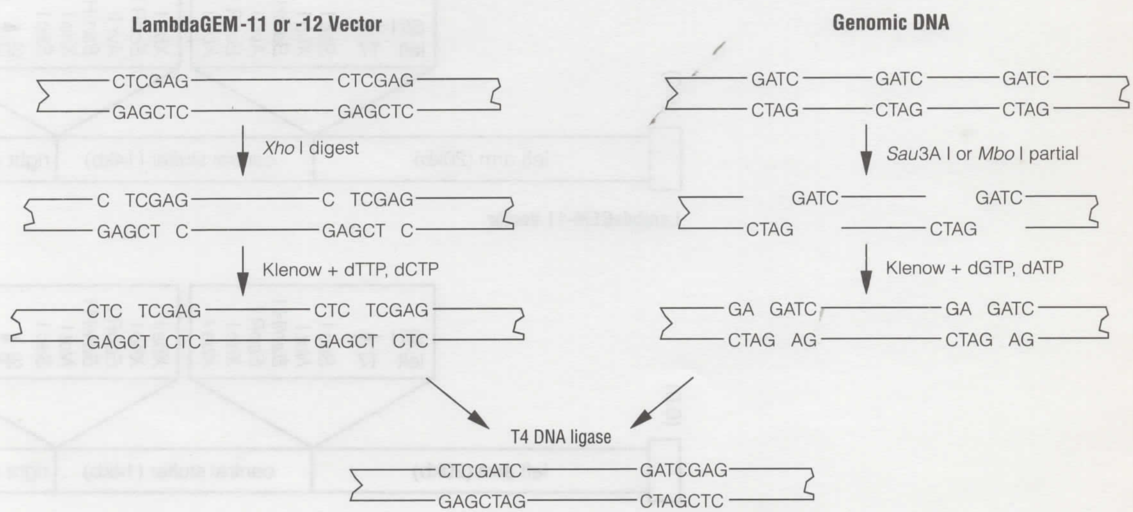

Figure 2. *Xho* I half-site arms cloning strategy.

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(continued)

and -12 vectors contain no pBR322 sequences. Thus, the few nonrecombinant plaques that do appear (1 in 10^5) will not hybridize with pBR322-derived probes.

The LambdaGEM-12 vector is derived from the LambdaGEM-11 vector and contains identical features, with the exception that the multiple cloning site contains a *Not* I rare-cutting site and lacks an *Avr* II site. Lambda cloning vectors are also available as uncut DNA. A protocol for the preparation of arms from uncut lambda vectors is included in Section V, pg. 186.

Table 1. Features and Applications of Lambda Genomic Cloning Vectors.

Lambda Vector	Cloning Sites	Non-Recombinant Background	*Sfi* I High Resolution Restriction Mapping	SP6/T7 Promoters/ Genomic Walking	Genomic DNA Fractionation Required
LambdaGEM®-11 Vector	*Sac* I, *Xho* I *Bam*H I, *Avr* II *Eco*R I, *Xba* I	*Spi* Selection	Yes	Yes	Yes
LambdaGEM®-11 *Bam*H I Arms	*Bam*H I (arms are also compatible with *Bcl* I, *Bgl* II, *Mbo* I and *Sau*3A I digested genomic DNA fragments)	Ultra-low <100 pfu/µg vector	Yes	Yes	Yes
LambdaGEM®-11 *Xho* I Half-Site Arms	Partially filled in *Xho* I (arms are also compatible with *Bcl* I, *Bgl* II, *Mbo* I and *Sau*3A I, digested, partially filled in genomic DNA fragments)	Ultra-low <100 pfu/µg vector	Yes	Yes	No
LambdaGEM®-12 Vector	*Sac* I, *Not* I, *Bam*H I, *Eco*R I, *Xho* I, *Xba* I	*Spi* Selection	Yes	Yes	Yes
LambdaGEM®-12 *Xho* I Half-Site Arms	Partially filled in *Xho* I (arms are also compatible with *Bcl* I, *Bgl* II, *Mbo* I and *Sau*3A I digested, partially filled in genomic DNA fragments)	Ultra-low <100 pfu/µg vector	Yes	Yes	No
EMBL3 Vector	*Sal* I, *Bam*H I, *Eco*R I	*Spi* Selection	No	No	Yes
EMBL3 *Bam*H I Arms	*Bam*H I (arms are also compatible with *Bcl* I, *Bgl* II, *Mbo* I, and *Sau*3A I digested genomic DNA fragments)	Ultra-low <100 pfu/µg vector	No	No	Yes
EMBL4 Vector	*Eco*R I, *Bam*H I, *Sal* I	*Spi* Selection	No	No	Yes

Genomic Cloning and Mapping

II. Partial Digestion and Size Fractionation of Genomic DNA

Genomic DNA can be isolated from a specific source using a variety of techniques (5,6). Once high molecular weight DNA has been successfully isolated, it is necessary to reduce the size range further (usually by restriction enzyme digestion) to ensure a successful genomic library construction.

Restriction digestion conditions should be optimized on a small scale (Section A, below) before performing large-scale digestion of genomic DNA for cloning (Section B, pg. 181). Partially digested DNA to be cloned into *Bam*H I arms of EMBL3 or LambdaGEM®-11 or -12 vectors should be further size-fractionated to remove fragments less than 14kb. This size fractionation minimizes the chance of cloning two or more DNA fragments into the same site since the maximum insert size is approximately 23kb. Size fractionation may be accomplished by sucrose gradient centrifugation (7), NaCl density gradient centrifugation (8), or by preparative agarose gel electrophoresis (9) followed by electroelution from the gel. Fragments to be cloned into the LambdaGEM-11 or LambdaGEM-12 vectors by the *Xho* I half-site strategy require no further size fractionation.

Reagents Needed

- *Sau*3A I 10X buffer (pg. 188)
- 1mg/ml acetylated BSA
- 0.5M EDTA
- sample buffer (pg. 188)
- TE-saturated phenol/chloroform (pg. 188)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)

A. Optimization of Restriction Endonuclease Digestion

In order to establish the optimum enzyme concentration to generate a certain size range of fragments (15-23kb), the following small-scale reactions are performed. In this example, *Sau*3A I is used; however, other restriction enzymes may be substituted.

1. Prepare the following buffers:

Dilution buffer:

*Sau*3A I 10X buffer (pg. 188)	0.15ml
1mg/ml acetylated BSA	0.15ml
H_2O	1.20ml
total volume	1.50ml

Table 2. Optimization of *Sau*3A I Partial Digestion of Genomic DNA.

Tube#	Preparation	Dilution
1	10µl *Sau*3A I + 140µl dilution buffer	1/15
2	10µl 1/15 dilution + 90µl dilution buffer	1/150
3	10µl 1/150 dilution + 10µl dilution buffer	1/300
4	10µl 1/150 dilution + 30µl dilution buffer	1/600
5	10µl 1/150 dilution + 50µl dilution buffer	1/900
6	10µl 1/150 dilution + 70µl dilution buffer	1/1200
7	10µl 1/150 dilution + 90µl dilution buffer	1/1500
8	10µl 1/150 dilution + 110µl dilution buffer	1/1800
9	10µl 1/150 dilution + 190µl dilution buffer	1/3000
10	10µl 1/150 dilution + 290µl dilution buffer	1/4500

Table 3. Final Enzyme Concentrations for *Sau*3A I Partial Digestion.

Tube#	Final Enzyme Concentration (u/µg)
1	1
2	0.1
3	0.05
4	0.025
5	0.015
6	0.0125
7	0.0100
8	0.0085
9	0.0050
10	0.0035

II. Partial Digestion and Size Fractionation of Genomic DNA

(continued)

Assay buffer:

genomic DNA (1µg/µl)	10µl
*Sau*3A I 10X buffer	50µl
1mg/ml acetylated BSA	50µl
H_2O	340µl
total volume	450µl

2. Prepare the following dilutions on ice (Table 2) using *Sau*3A I (at 3u/µl).

3. After the dilutions have been made, assemble small-scale digestion reactions in separate tubes (Table 3). This is done by numbering 10 individual tubes, adding 45µl of assay buffer to each tube, and then adding 5µl of the appropriate dilution prepared in Step 2.

4. Incubate the 10 digestion reactions at 37°C for 30 minutes. Stop the reactions by adding 1µl of 0.5M EDTA and 10µl of sample buffer (pg. 188).

5. Load approximately 20µl of each reaction on a 0.4% agarose gel along with DNA markers (Lambda digested with *Hin*d III). Run the gel at 2 volts/cm for 16-20 hours or until the bromophenol blue has just migrated off the gel.

6. Photograph the gel and determine the amount of enzyme needed to produce the maximum intensity of fluorescence in the desired size range. The intensity of fluorescence is related to the mass distribution of the DNA. To obtain the maximum number of molecules in this size range for library construction, use half of the amount of enzyme that produces the maximum amount of fluorescence (10).

B. Large-Scale Preparation of Partially Digested Genomic DNA

1. Using the optimized conditions determined in Step A.6, carry out a large-scale reaction with 50-100µg of high molecular weight genomic DNA. The DNA concentration, time, and temperature should be identical to those used in the small-scale reactions.

   Note: As discussed in Step A.6, use half the number of units of *Sau*3A I/µg DNA to optimize sequence representation of molecules in the desired size range.

2. Determine the size distribution of the digestion products by removing a small aliquot of the DNA (0.5µg) and analyzing by electrophoresis through a 0.4% agarose gel.

3. If the digestion is adequate, extract with 1 volume of TE-saturated phenol/chloroform (pg. 188). Mix by gently inverting for several minutes and centrifuge at 12,000 x g for 5 minutes.

4. Transfer the upper, aqueous phase to a fresh tube and repeat Step B.3.

5. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Mix and centrifuge as in Step B.3.

6. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume of 7.5M ammonium acetate followed by 2 volumes of ethanol. Mix and leave at –20°C for 30 minutes.

7. Centrifuge at 12,000 x g for 10 minutes. Remove the supernatant, and rinse the pellet with 70% ethanol. Drain the tube and dry the pellet under vacuum. Resuspend the pellet in 500µl of TE buffer (pg. 188). Store the DNA at –20°C.

Genomic Cloning and Mapping

III. Low Background Ligation and Cloning Procedures for *Bam*H I and *Xho* I Half-Site Arms

The procedures described below may be used for cloning genomic DNA fragments into either *Bam*H I or *Xho* I half-site arms made from Promega's EMBL3, LambdaGEM®-11 and LambdaGEM®-12 vectors. Fragments to be cloned into *Xho* I half-site arms must undergo the partial fill-in reaction described in Section III.A, below, before ligation. Fragments to be cloned into *Bam*H I arms can be ligated directly (see Section III.B, below) following size-fractionation (7, 8, 9).

Reagents Needed

Instructions for preparing these buffers and media are provided on pg. 188.

- Klenow fragment
- fill-in 10X buffer
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- ethanol (100% and 70%)
- T4 DNA ligase
- ligase 10X buffer
- Packagene® packaging extract
- phage buffer
- chloroform
- LB medium
- LB plates
- TB top agar

A. Partial Fill-In Reaction for Cloning into *Xho* I Half-Site Arms

1. Standard Reaction:

partially digested genomic DNA	10µg
fill-in 10X buffer containing dGTP and dATP (pg. 188)	5µl
Klenow fragment	1u/1µg DNA
H_2O	to final volume 50µl

Incubate at 37°C for 30 minutes.

2. Extract twice with 1 volume of TE-saturated phenol/chloroform (pg. 188). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

3. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 2. Repeat this step.

4. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M ammonium acetate. Add 2 volumes of ethanol and leave at –70°C for 30 minutes. Centrifuge at 12,000 x g for 15 minutes.

5. Carefully pour off the supernatant, wash the pellet with 1ml 70% ethanol, dry briefly in a vacuum desiccator, and resuspend in 20µl H_2O (approximately 0.5µg/µl). Determine the exact DNA concentration by absorption spectroscopy.

B. Ligation of Positive Control Insert DNA

1. The day before the experiment, preferably late in the day, start an overnight culture of LE392 or KW251 (see Note 1) by inoculating a single colony into 50ml of LB medium (pg. 189) supplemented with 0.5ml of 20% maltose and 0.5ml of 1M $MgSO_4$. Shake overnight at 37°C and early the next day store at 4°C. Alternatively, inoculate 50ml of supplemented LB medium with 1ml of an overnight culture of cells, shake at 37°C, and store at 4°C once the $O.D._{600}$ has reached 0.6.

2. Prepare the ligation reaction:

(**Note:** To determine the optimal ligation conditions for a genomic DNA insert, see Step III.E, pg. 183.)

lambda vector *Bam*H I or *Xho* I half-site arms	0.5µg
test insert (for *Bam*H I arms) or half-site text insert (for *Xho* I half-site arms)	0.5µg
ligase 10X buffer (pg. 188)	0.5µl
T4 DNA ligase (1 Weiss unit)	0.5µl
H_2O	to final volume 5µl

To test the background due to the vector arms alone, prepare the above reaction, but omit the test insert.

3. Incubate the ligation mixture(s) for 3 hours at room temperature for *Bam*H I arms or overnight at 4°C for *Xho* I half-site arms.

III. Low Background Ligation and Cloning Procedures for *Bam*H I and *Xho* I Half-Site Arms

(continued)

C. Packaging of Ligated DNA using the Packagene® *in vitro* Packaging System

1. Thaw the Packagene extract on ice. (**Note:** The volume of the Packagene extract is 50µl.)

2. Add the entire ligation reaction (Step B.3) to the extract, and mix by gently tapping the bottom of the tube several times. (To determine the efficiency of the Packagene reaction alone, add 0.5µg of the provided concatemeric lambda cI857 *Sam*7 DNA directly to the extract and mix.)

3. Incubate at 22°C for 2 hours.

4. To the packaging mix (55µl), add phage buffer (pg. 189) to 0.5ml and 25µl of chloroform (vector DNA concentration = 1µg/ml, if 0.5µg was packaged). Mix gently by inversion and allow the chloroform to settle to the bottom of the tube. The packaged phage can then be stored at 4°C for up to 3 weeks, although the titer may drop several fold under these conditions.

D. Titration of Packaged Phage on LB Plates

1. Make appropriate dilutions of the packaging extracts (from Step C.4) in phage buffer. As a general guideline, an appropriate dilution for recombinant phage is 1/1,000 or 1/10,000.

2. Add 100µl of the diluted phage to 100µl of prepared LE392 or KW251 bacteria (Step B.1) and allow the phage to adsorb for 30 minutes at 37°C. The lambda cI857 *Sam* 7 control DNA must be used with a host that contains the *sup*F58 mutation. Both LE392 and KW251 contain this mutation.

3. Add 3ml molten (45°C) TB top agar. Vortex gently and immediately pour onto LB plates. Allow the top agar to harden and incubate inverted at 37°C overnight. (Best results are obtained by using fresh plates which have been allowed to dry overnight at room temperature so that they lose excess moisture.)

4. Count the number of plaques and calculate the titer of the phage. For example: if there are 20 plaques on a plate made from a 1/10,000 dilution, that means there are 20 x 10,000 x 10 = 2 x 10^6 plaque forming units (pfu) per milliliter of the original packaging extract. (The last 10 of the calculation converts the 0.1ml of the packaging extract which was plated to a per milliliter basis). Since the vector DNA being packaged was at 1µg/ml, the efficiency of the arms is calculated to be 2 x 10^6 recombinants per microgram.

E. Determination of Optimal Ligation Conditions of DNA Inserts with Lambda Vector *Bam*H I and *Xho* I Half-Site Arms

1. To determine the optimal ligation conditions for a genomic DNA insert, proceed with the small scale reactions described below and in Table 4 (pg. 184). Determining optimal ligation conditions is optional, but is encouraged if sufficient amounts of the genomic insert are available.

   We recommend that the molar concentration of the lambda vector remain constant while adjusting the amount of insert DNA. In the examples given, the molar ratio of lambda arms (43kb) to genomic insert (average 15kb) varies between approximately 1:3 and 1:0.5. A tube lacking insert DNA is also used to determine background levels of religated arms.

2. Ligate and package DNA as indicated in Steps B-D, above.

3. Compare the titers from the various plates to determine the optimal relative concentrations of vector arms and DNA insert. The yield of recombinant plaques (from tubes B to D, Table 4) should be approximately 100 to 1,000-fold greater than the yield of nonrecombinant plaques on the control plate (from tube A, Table 4). Prepare a large scale reaction based on the optimum ratio of insert to vector DNA determined above. The amount of ligated DNA added to a Packagene extract in a large scale reaction may range between 0.5µg-5µg (suspended in a volume of 5-10µl). As little as 50ng of ligated DNA may be packaged, but the resulting phage titers will be considerably reduced.

Genomic Cloning and Mapping

III. Low Background Ligation and Cloning Procedures for *Bam*H I and *Xho* I Half-Site Arms

(continued)

Table 4. Optimization of Ligation of Genomic Inserts with Lambda *Bam*H I and *Xho* I Half-Site Arms.

	TUBE A	TUBE B	TUBE C	TUBE D
Vector DNA (500ng/µl; 1µg = 0.035pmole)	2µl	→	→	→
Insert DNA (300ng/µl; 100ng = 0.01pmole)	—	3µl	2µl	0.5µl
Ligase 10X Buffer	1µl	→	→	→
H_2O	6µl	3µl	4µl	5.5µl
T4 DNA Ligase (1-2 Weiss units)	1µl	→	→	→

F. Troubleshooting

The following suggestions can be used as guidelines for analyzing problems occurring in the construction of genomic libraries.

1. Fresh (or freshly thawed) ligase 10X buffer is recommended. The activity of the T4 DNA ligase can be evaluated by ligation of the *Bam*H I test insert to itself and noting a shift in mobility (up) on a low percentage agarose gel. The activity of Klenow can be determined by filling in an appropriate DNA fragment (i.e., digesting a plasmid with a restriction enzyme creating a 5′ overhang) with a labeled dNTP (9). Incorporation can be monitored by TCA precipitation.

2. If low titers are observed, we recommend the following:
   a. If utilizing LE392 as the primary host strain, KW251 may be used as an alternative (see Note 1).
   b. A mixing experiment may be performed in which a small aliquot of genomic insert (pre-fill reaction DNA) is mixed either with the positive control insert or lambda DNA markers. This mixture is then ligated under normal experimental conditions. A control tube containing no genomic insert and only the secondary DNA is also ligated. The samples are then loaded on a low percentage agarose gel. Inhibition of ligation efficiencies can then be noted by comparison of the two distinct samples. If ligation inhibition is observed, the genomic DNA sample should be re-extracted with phenol/chloroform, extracted with chloroform alone, and then precipitated with ethanol.
   c. Polysaccharide contamination (observed usually in plant or bacterial genomic DNA samples) will inhibit the Klenow fill-in or the ligation reaction. The recommended procedure for polysaccharide removal is chloroform extraction in the presence of 1% cetyltrimethylammonium bromide (CTAB) (10).
   d. The genomic DNA inserts (pre-fill) may be ligated to themselves and observed on a low percentage agarose gel. If no apparent shift in mobility is noted, it is possible that the sample has been contaminated with an exonuclease, therefore not allowing an efficient fill-in reaction or ligation to the provided lambda arms.

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III. Low Background Ligation and Cloning Procedures for *Bam*H I and *Xho* I Half-Site Arms

(continued)

Notes:

1. Two different bacterial host strains, LE392 and KW251, are provided with Promega's pre-digested vectors. Due to the low background of Promega's *Bam*H I and *Xho* I half-site arms (<100pfu/µg vector), *Spi* selection is not necessary, thus allowing screening to be carried out utilizing permissive host strains.

   LE392: F^-, *hsd*R514 (r_k^-, m_k^-), *sup*E44, *sup*F58, *lac*Y1 or Δ(*lac*IZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55, λ^-. The bacterial strain LE392 is a permissive host strain that can be utilized for most cloning experiments.

   KW251: F^-, *sup*E44, *sup*F58, *gal*K2, *gal*T22, *met*B1, *hsd*R2, *mcr*B1, *mcr*A^-, *arg*A81:*Tn*10, *rec*D1014. The bacterial strain KW251 is also a permissive host strain. It has been reported that certain eukaryotic recombinants are unable to grow on or can be biased by *rec*$^+$ host strains (i.e., NM538, NM539, LE392) (11). If low cloning efficiencies are noted using conventional host strains, KW251 may be used as an alternative host. KW251 should be maintained in the presence of tetracycline.

   When the EMBL3, EMBL4, LambdaGEM-11 and LambdaGEM-12 vectors are supplied as uncut DNA, the LE392 (permissive), NM538 (permissive) and NM539 (restrictive) host strains are provided (8). NM539 is required when *Spi* selection for recombinants is to be used. The latter two strains are *rec*$^+$, which can lead to the deletion or rearrangement of potential clones by recombination (see pg. 412 for more information).

   NM538: *sup*F, *hsd*R (r_k^-, m_k^+).

   NM539: *sup*F, *hsd*R (r_k^-, m_k^-) (P2).

2. Typically, recombinant efficiencies of 7 x 10^6 to 2 x 10^7 are achieved when using the positive control DNA insert provided. The background of nonrecombinants is typically less than 100pfu/µg in the absence of the insert. The positive control insert provided with the LambdaGEM-11 and -12 *Xho* I half-site arms is partially filled in.

IV. Preparation of Lambda Lysates and Isolation of Lambda DNA

The protocols listed below are provided in the Nucleic Acid Detection, Purification and Labeling chapter (pgs. 136-140).

A. Plate Method for Phage Lysate Preparation

B. Liquid Culture Method for Phage Lysate Preparation

C. Isolation of Lambda DNA with LambdaSorb® Phage Adsorbent

D. Miniprep Isolation of Recombinant Lambda DNA

Genomic Cloning and Mapping

V. Preparation of Lambda Vector Arms for Genomic Cloning

Lambda vectors can be obtained as uncut DNA. Procedures are provided below for restriction digestion of vector DNA and ligation with the DNA fragments to be inserted.

Reagents Needed

Instructions for preparing these buffers and media are provided on pg. 188.

- appropriate restriction enzymes and 10X buffers
- acetylated BSA
- calf intestinal alkaline phosphatase
- alkaline phosphatase 10X buffer
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- T4 DNA ligase
- ligase 10X buffer
- Packagene® packaging extract
- phage buffer
- chloroform
- LB medium
- LB plates
- LB top agar

A. Restriction Digestion of Vector Arms

Digest 10-50µg of vector DNA with a 4- to 5-fold excess of the restriction enzyme for 2 hours. Check that the reaction has gone to completion by electrophoresis of a sample (0.3µg) on an agarose minigel. To lower the background of nonrecombinants, perform a double-digest if possible. For example, if preparing LambdaGEM-12 *Not* I arms, digest with both *Eco*R I and *Not* I in *Eco*R I digestion buffer.

Sample Reaction:

lambda vector DNA at 0.5µg/µl	25µl
*Eco*R I 10X buffer	25µl
acetylated BSA at 1mg/ml (optional)	25µl
*Eco*R I at 20u/µl	3µl
Not I at 20u/µl	3µl
H_2O	to final volume 250µl

Incubate at 37°C for 2 hours.

If the phosphate groups on the arms are to be removed, proceed directly to Step B; if not, proceed to Step C.

B. Dephosphorylation of Vector Arms

This step is optional, depending on the cloning strategy being utilized. Dephosphorylating the ends of the cut vector will further reduce the background of nonrecombinant plaques obtained.

Sample Reaction:

*Eco*R I-*Not* I digested lambda vector DNA (from Step I)	250µl
alkaline phosphatase 10X buffer (pg. 188)	40µl
calf intestinal alkaline phosphatase	20u
H_2O	to final volume 400µl

Incubate for 1 hour at 37°C. Proceed to Step C.

C. Inactivation of Restriction Enzymes and Alkaline Phosphatase

1. Extract twice with 1 volume of TE-saturated phenol/chloroform. Vortex gently and centrifuge at 12,000 x g for 5 minutes.

2. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

3. Transfer the upper, aqueous phase to a fresh tube. Add 0.5 volume of 7.5M NH_4OAc. Add 2 volumes of ethanol and leave at –70°C for 30 minutes. Centrifuge at 12,000 x g for 15 minutes.

4. Carefully pour off the supernatant, wash the pellet with 1ml of 70% ethanol, dry briefly in a vacuum desiccator and resuspend in 25µl of H_2O (approximately 0.5µg/µl). Determine the exact DNA concentration by absorption spectroscopy.

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V. Preparation of Lambda Vector Arms for Genomic Cloning

(continued)

D. Determination of the Optimal Ligation Conditions for DNA Inserts with Lambda Vectors (Optional)

1. To determine the optimal ligation conditions for a genomic DNA insert, proceed with the following small scale reactions (Table 5). We recommend that the molar concentration of the lambda vector remain constant while adjusting the amount of insert DNA. A tube lacking insert DNA is also used to determine background levels of religated arms.

2. Ligate and package DNA as indicated on pg. 182, Steps A.1-B.3.

3. Compare the titers from the various plates to determine the optimal relative concentrations of vector arms and DNA insert. The yield of recombinant plaques (from tubes B to E, Table 5) should be approximately 100- to 1,000-fold greater than the yield of nonrecombinant plaques on the control plate (from tube A, Table 5). Prepare a large scale reaction based on the optimum ratio of insert to vector DNA determined above. The amount of ligated DNA added to a Packagene extract in a large scale reaction may range between 0.5µg-5µg (suspended in a volume of 5-10µl). As little as 50ng of ligated DNA may be packaged, but the titers will be considerably reduced.

Table 5. Optimization of Lambda Vector: Genomic Insert Ligation.

	TUBE A	B	C	D	E
Vector DNA (0.5µg/µl; 1µg = 0.035pmole)	1µl →				
Insert DNA (50ng/µl; 100ng = 0.01 pmole)	—	6µl	4µl	2µl	0.5µl
ligase 10X buffer	1µl →				
H_2O	7µl	1µl	3µl	5µl	6.5µl
T4 DNA ligase (1-2 Weiss units)	1µl →				

Table 6. Restriction Enzyme 10X Buffers.

	Tris-HCl (mM)	pH	NaCl (mM)	$MgCl_2$ (mM)	DTT (mM)
Avr II	100	7.4	500	100	—
*Bam*H I	100	7.9	500	100	10
*Eco*R I	900	7.5	500	100	—
Not I	60	7.9	1,500	60	10
Sac I	60	7.5	60	60	10
Xba I	60	7.9	1,500	60	10
Xho I	60	7.9	1,500	60	10

Genomic Cloning and Mapping

V. Preparation of Lambda Vector Arms for Genomic Cloning

(continued)

Notes:

1. In order to perform a restriction digestion with two different restriction enzymes, it is necessary to choose a 10X buffer in which both are active. Since *Eco*R I and *Bam*H I are susceptible to star activity, these enzymes should be used in their own buffers whenever possible.

   Tables 6 and 7 illustrate the appropriate 10X buffers recommended by Promega and the effect of NaCl concentration on enzyme activity of those restriction enzyme sites found in the polylinker of the LambdaGEM-11 and -12 vectors.

2. Two different bacterial strains (NM538 and NM539) are provided with lambda vectors supplied as uncut DNA. NM538 (*sup*F, *hsd*R) is a permissive host strain. It allows both recombinant and parental phage to form plaques. NM539 (*sup*F, *hsd*R, P2*cox*) is a restrictive host, hence recombinant phages are strongly favored over parentals on such a strain. This selection process is due to the inability of *gam*⁺ phages to form plaques on *E. coli* lysogenic for phage P2 (1). The EMBL3 and EMBL4 and LambdaGEM-11 and -12 vectors include a functional *gam* gene in the central stuffer fragment. Parental sequence genomes (*gam*⁺) will be among the products of the ligation reaction if modification of the lambda vector arms is complete. However, they can be effectively discriminated against by propagating the library on NM539.

Table 7. Effect of NaCl Concentration (mM) on Activity of Selected Restriction Enzymes.

	0	50	100	150
Avr II	++	++	++	+
*Bam*H I	–	++	++	++
*Eco*R I	–	++	++	++
Not I	–	++	++	++
Sac I	++	++	+	–
Xba I	–	++	++	++
Xho I	–	++	++	++

++ indicates >50% activity
\+ indicates 10%-50% activity
\- indicates <10% activity

Composition of Solutions

***Sau*3A I 10X buffer:**

100mM Tris-HCl, pH 7.5
1M NaCl
70mM $MgCl_2$

Sample buffer:

38% sucrose
0.1% bromophenol blue
67mM EDTA

Alkaline phosphatase 10X buffer:

500mM Tris-HCl, pH 9.0
10mM $MgCl_2$
1mM $ZnCl_2$
10mM spermidine

TE buffer*:

10mM Tris-HCl, pH 8.0
1mM EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Ligase 10X buffer:

300mM Tris-HCl, pH 7.8
100mM $MgCl_2$
100mM DTT
10mM ATP

Note: We recommend that the ligase 10X buffer be stored frozen in aliquots at –20°C to avoid multiple freeze-thaw cycles. Degradation of ATP in the ligation buffer is the most common reason for failure of simple ligations.

Fill-in 10X buffer:

0.5M Tris-HCl, pH 7.2
0.1M $MgSO_4$
1mM DTT
500µg/ml acetylated BSA
10mM dATP
10mM dGTP

*Buffer tablets available from Promega.

Promega

V. Preparation of Lambda Vector Arms for Genomic Cloning

(continued)

Phage buffer:

20mM	Tris-HCl, pH 7.4
100mM	NaCl
10mM	$MgSO_4$

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust pH to 7.5 with NaOH. Autoclave.

LB plates:

Add 15 grams of Bacto-agar to one liter of LB medium. Autoclave. Pour 30-35ml of medium into 100mm petri dishes. If necessary, flame the surface of the medium with a bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month). If plates are excessively wet, place them at room temperature the evening prior to plating.

TB top agar (100ml):

1.0g	Bacto-tryptone
0.5g	NaCl
0.8g	Bacto-agar

Microwave or autoclave to melt the agar. When the solution has cooled to 60°C, add 1ml of 1M $MgSO_4$.

VI. High Resolution Restriction Mapping of Lambda Inserts with the *Sfi* Linker Mapping System

A. Description

The *Sfi* linker mapping system is designed for the high resolution restriction mapping of genomic inserts cloned in the LambdaGEM®-11 or LambdaGEM®-12 vectors. The strategy is based on the asymmetrical labeling of either end of the insert, as shown in Figure 3 (pg. 190). LambdaGEM-11 or -12 recombinant DNA is first digested to completion with *Sfi* I. The *Sfi* I restriction sites allow most inserts to be excised as a single fragment, since the 8-base *Sfi* I recognition sequence occurs infrequently in genomic DNA (statistically once every 65,536bp).

Sfi I recognizes the interrupted palindrome GGCCNNNN/NGGCC and cleaves within the central unspecified sequence, leaving a 3-base 3′ overhang (12). Any of the four possible bases can occupy the 5-base central region which becomes the overhanging termini. The flanking *Sfi* I sites which can be in the LambdaGEM-11 and -12 vectors have been designed in an asymmetric fashion, so that the site on the left is distinct from the site on the right. Therefore, radiolabeled linkers complementary to either the left or right *Sfi* I termini can be ligated separately to the *Sfi* I excised genomic DNA. Once the insert has been asymmetrically labeled, a high resolution restriction map can be generated by partial digestion with a frequently cutting restriction endonuclease such as *Sau*3A I. By analyzing the fragment lengths generated in relationship to the provided DNA standards, the position of each restriction site within the recombinant molecule can be determined. By comparison of the data from both ends, a comprehensive restriction map of the recombinant lambda DNA can be easily determined.

The mapping resolution of this method is an order of magnitude greater than conventional cos-site oligo labeling, since only the ends of the insert are labeled instead of the ends of the 20kb and 9kb arms of the vector. Variable results generated from inaccurate size estimates of large restriction size fragments and anomalous bands resulting from the fusion of the insert with a vector fragment are eliminated with this system. An additional advantage of this method is the larger number of restriction sites which can be mapped, and thus greater resolution achieved, by the use of frequently cutting enzymes.

Reagents to be Supplied by the User

- TE-saturated phenol/chloroform (pg. 193)
- chloroform:isoamyl alcohol (24:1)
- 7.5M ammonium acetate
- kinase 10X buffer (pg. 193)
- ligase 10X buffer (pg. 193)
- [γ-^{32}P]ATP
- stop mix (pg. 193)

Genomic Cloning and Mapping

VI. High Resolution Restriction Mapping of Lambda Inserts with the *Sfi* Linker Mapping System

(continued)

High Resolution *Sfi* Mapping Strategy using LambdaGEM-11 or LambdaGEM-12

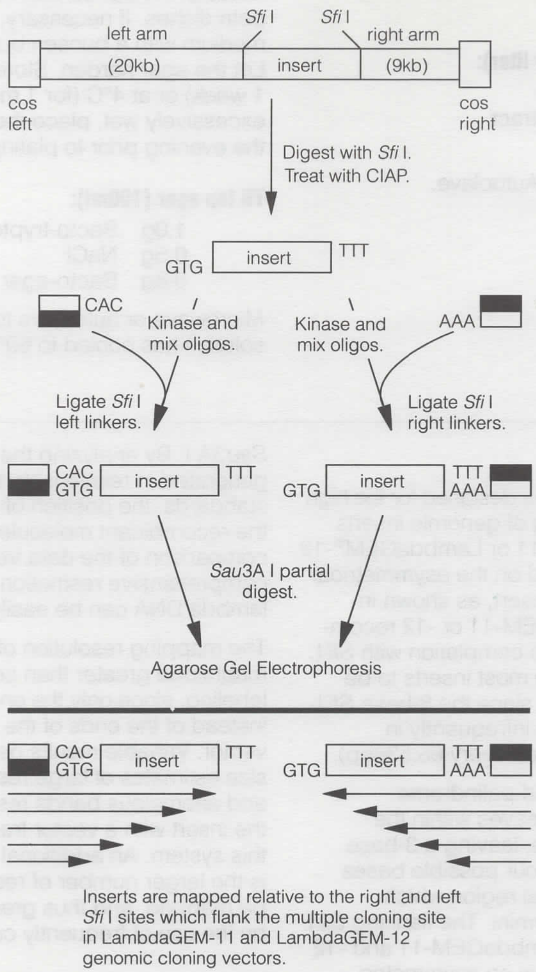

Figure 3. *Sfi* I linker mapping strategy using LambdaGEM recombinants.

B. *Sfi* I Digestion and Dephosphorylation of Recombinant DNA

DNA isolated from phage mini-preps using LambdaSorb® phage adsorbent is suitable for this procedure. Digest to completion 1-5μg of the LambdaGEM-11 or -12 recombinant containing the sequence of interest with *Sfi* I in the presence of calf intestinal alkaline phosphatase (CIAP). For unknown reasons, the ligation of *Sfi* linkers to the digested vector is very inefficient unless the vector DNA has been dephosphorylated. To facilitate the ligation of the *Sfi* linkers, the restriction digest is performed concurrently in the presence of CIAP. A typical reaction is given below.

1. Mix the following reagents in a 1.5ml microcentrifuge tube:

LambdaGEM recombinant	1μg
Sfi I 10X buffer (pg. 193)	2μl
bovine serum albumin at 1mg/ml	2μl
Sfi I	8u
calf intestinal alkaline phosphatase	1u
H_2O	to final volume 20μl

2. Incubate at 50°C for 1 hour.

3. Inactivate the enzymes by adding 1 volume of TE-saturated phenol/chloroform (pg. 193). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

4. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.

5. Transfer the aqueous phase to a fresh tube. Add 0.5 volume of 7.5M NH_4OAc. Add 2 volumes of ethanol and mix. Leave at –70°C for 30 minutes. Centrifuge at 12,000 x g for 15 minutes.

6. Resuspend the DNA in H_2O at a concentration of 250ng/μl.

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VI. High Resolution Restriction Mapping of Lambda Inserts with the *Sfi* Linker Mapping System

(continued)

C. Radiolabeling and Mixing of Oligonucleotides

Three single-stranded oligonucleotides are required to asymmetrically label the two *Sfi* termini: an *Sfi* I left oligo (5′-CCGTGCTGCCAC-3′), an *Sfi* I right oligo (5′-CCGTGCTGCAAA-3′), and an oligonucleotide which is complementary in sequence to a portion of both the left and right *Sfi* oligos (5′-GCAGCACGG-3′). The complementary oligo is radiolabeled separately and mixed with the unlabeled left or right oligo to form a double-stranded linker with *Sfi* I sticky ends.

1. Mix the following reagents in a 1.5ml microcentrifuge tube:

complementary oligo (20pmol)	2µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10µCi/µl, 20pmol)	6µl
kinase 10X buffer (pg. 193)	1µl
T4 polynucleotide kinase (5-10u/µl)	1µl

2. Incubate at 37°C for 10 minutes.

3. Inactivate the kinase at 100°C for 1 minute. Briefly spin in a microcentrifuge to bring down any condensation.

4. In separate 1.5ml microcentrifuge tubes, combine the *Sfi* I left or *Sfi* I right oligos with the labeled complimentary oligo (C oligo) from above:

5µl labeled C oligo	(10pmol)
5µl *Sfi* I left oligo	(10pmol)
10µl *Sfi* I left linker	(10pmol)

5µl labeled C oligo	(10pmol)
5µl *Sfi* I right oligo	(10pmol)
10µl *Sfi* right linker	(10pmol)

D. Ligation of *Sfi* Linkers to *Sfi* I Digested Recombinant DNA

The DNA insert which has been prepared in Step B is ligated separately to the radiolabeled *Sfi* left or right linker as described below. Note that 1pmol of *Sfi* linker is sufficient to label 50ng (approximately 1.5fmol) of recombinant LambdaGEM-11 or -12 DNA, which is an amount sufficient for 1 gel lane in an overnight exposure under the conditions described here. To label additional amounts of DNA, simply replace the H_2O in the reaction with more DNA, while maintaining a 1pmol to 1fmol (1,000:1) ratio of linker to phage DNA. Up to 0.25µg of phage DNA can be ligated to 5pmoles of linker in a 10µl reaction volume, and if necessary the reaction can be scaled-up proportionately.

1. Mix the following reagents in a 1.5ml microcentrifuge tube:

LambdaGEM-11 or -12 [*Sfi* I + CIAP] DNA (250ng) from Step B	1µl
Sfi I left or right linker (5pmol) from Step C	5µl
ligase 10X buffer (pg. 193)	1µl
T4 DNA ligase (2.5u/µl)	1µl
H_2O	to final volume 10µl

2. Incubate at room temperature for 3 hours.

3. Heat inactivate the ligase by incubating at 65°C for 10 minutes. Briefly spin in a microcentrifuge to bring down any condensation.

E. Partial Digestion with a Frequent Cutting Restriction Enzyme

The asymmetrically labeled insert DNA is now ready for partial restriction digestion with a frequently cutting enzyme such as *Sau*3A I. To find reaction conditions that produce all possible partial restriction fragments, the enzyme concentration should be titrated versus DNA concentration. This is determined empirically by dilution of the restriction enzyme. Several trial reactions may be required to bracket the optimal enzyme to DNA ratio, as described below.

1. Mix the following reagents with the indicated amounts of enzyme in separate 1.5ml microcentrifuge tubes:

radiolabeled DNA (50ng) from Section D	2µl
*Sau*3A I 10X buffer (pg. 193)	2µl
*Sau*3A I (0.4u/µl, 0.2u/µl, 0.1u/µl, 0.05u/µl, 0.025u/µl)	1µl
H_2O	to final volume 20µl

2. Incubate at 37°C for 30 minutes.

3. Stop the reaction by adding 2µl of stop mix.

VI. High Resolution Restriction Mapping of Lambda Inserts with the *Sfi* Linker Mapping System

(continued)

F. Gel Electrophoresis and Autoradiography

1. Load samples and labeled DNA size markers (Section G, below) onto a 0.4% agarose gel and run at 1.5 volts/cm for 12-24 hours. Before electrophoresis, lambda DNA markers should be heated to 65-70°C for 5 minutes and then cooled on ice to prevent annealing of cos sites. Either Tris-acetate or Tris-borate electrophoresis buffers may be used (9).

2. After electrophoresis, rinse the gel briefly in water.

3. Cut a piece of 3MM paper slightly larger than the gel. Cut two more pieces of 3MM paper and a piece of nylon transfer membrane (e.g., GeneScreen™* membrane) to the size of the gel. Pre-wet the nylon membrane in water for 20 minutes. (*GeneScreen is a registered trademark of DuPont NEN Research Products.)

4. Place the oversized piece of 3MM paper in a shallow tray. Place the gel on top of the paper and the wetted nylon membrane on top of the gel. Smooth out any bubbles.

5. Briefly soak the remaining pieces of 3MM filter paper in water and place on top of the nylon.

6. Place 2-5cm of blotting material, such as paper towels, on top of the 3MM filter paper. Finally, place an evenly distributed weight of 1-2kg on top.

7. After 2 hours, the gel should be compressed to approximately 1mm. Wrap the gel and nylon membrane with plastic wrap. Place a piece of X-ray film on the wrapped gel and place in an autoradiograph holder for 1-2 days at room temperature. If an intensifying screen is used (–70°C), exposure is complete within 5-7 hours.

G. Radiolabeling of DNA Size Markers

1. Mix the following reagents in a 1.5ml microcentrifuge tube:

Sfi mapping marker DNA (50ng)	1µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10µCi/µl)	3µl
kinase 10X buffer (pg. 193)	1µl
T4 polynucleotide kinase (5-10u/µl)	0.5µl
H_2O	4.5µl
final volume	10µl

2. Incubate at 37°C for 10 minutes.

3. Stop the reaction by adding 2µl of stop mix (pg. 193).

4. Load 10ng of radiolabeled marker in a gel lane. This amount is sufficient for visualizing after an overnight exposure without screens.

H. *Sfi* Linker Mapping Positive Control Protocol

The system is supplied with *Sfi* I digested and dephosphorylated LambdaGEM-11 DNA as a positive control to test the efficiency of the *Sfi* linker labeling procedure. The protocol is similar to that described above, and the appropriate steps from previous sections can be referred to for details.

1. Radiolabel and mix oligonucleotides as previously described in Section C, pg. 191.

2. Ligate *Sfi* linkers to *Sfi* I-CIAP treated positive control LambdaGEM-11 DNA as follows:

   a. Mix the following reagents in a 1.5ml microcentrifuge tube:

positive control DNA (50ng)	1µl
Sfi left or right linker (1pmol) from Step A.2	1µl
ligase 10X buffer (pg. 193)	1µl
T4 DNA ligase (2.5u/µl)	1µl
H_2O	6µl
final volume	10µl

   b. Incubate at room temperature for 3 hours.

   c. If a partial restriction digest is to be performed, inactivate the ligase reaction at 65°C for 10 minutes. Briefly spin in a microcentrifuge to bring down any condensation, and proceed to the partial restriction reaction detailed in Section E above. To visualize the radiolabeled insert, stop the reaction by adding 2µl of 10X stop mix and load onto an agarose gel for electrophoresis and autoradiography as detailed in Section F. *Sfi* linker labeling of the positive control yields a 14kb insert fragment. To confirm that the central insert has been labeled but the arms have not, the positive

VI. High Resolution Restriction Mapping of Lambda Inserts with the *Sfi* Linker Mapping System

(continued)

control can be kinased and run next to the *Sfi* linker labeled sample. The kinased positive control will show three labeled DNA fragments; a 20kb left arm, the 14kb central insert, and a 9kb right arm.

I. Sizes of Molecular Weight Markers for the *Sfi* I Linker Mapping System

Restriction digested and dephosphorylated lambda DNA is supplied with this system as ready-to-kinase molecular weight markers. The markers consist of a mixture of lambda cI857 individually digested with *Eco*R I, *Hin*d III, and *Xho* I and dephosphorylated with alkaline phosphatase. These markers consist of 16 fragments of the following sizes (in base pairs):

33,498	5,643
23,130	4,875
21,226	4,361
15,004	3,530
9,416	2,322
7,421	2,027
6,557	564
5,804	124

Composition of Solutions

***Sfi* I 10X buffer:**

100mM	Tris-HCl, pH 7.8
500mM	NaCl
100mM	$MgCl_2$
100mM	β-mercaptoethanol

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Kinase 10X buffer:

500mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
50mM	DTT
1mM	spermidine

Ligase 10X buffer:

300mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

***Sau*3A I 10X buffer:**

100mM	Tris-HCl, pH 7.5
1M	NaCl
70mM	$MgCl_2$

Stop mix:

2%	Ficoll
0.5%	SDS
50mM	EDTA
0.2%	orange G
or	
50%	glycerol
0.5%	SDS
100mM	EDTA
0.1%	bromophenol blue

Genomic Cloning and Mapping

VII. Restriction Mapping of Recombinant Lambda DNA with the LambdaMap™ System

A. Description

Lambda DNA is a 48.5kb molecule which is double-stranded except for its cohesive ends (cos sites) which are each 12-base single strands. The LambdaMap™ system takes advantage of this structural characteristic to provide an efficient scheme for the restriction mapping of recombinant clones (see Figure 4). This mapping strategy can be applied to wild type lambda and any lambda cloning vector. Included in the system are two synthetic 12-base oligonucleotides (ON-L, ON-R) which are complementary to their respective cos sites. Once kinased, these probes are hybridized to fragments of recombinant lambda DNA which have been partially digested with a restriction enzyme. The hybrids are then separated by agarose gel electrophoresis and visualized after autoradiography. By comparing the fragment lengths generated to the DNA standards provided, the position of each restriction site within the recombinant molecule can be determined. By comparison of the data of both hybrids, a comprehensive restriction map of the recombinant lambda DNA can easily be produced.

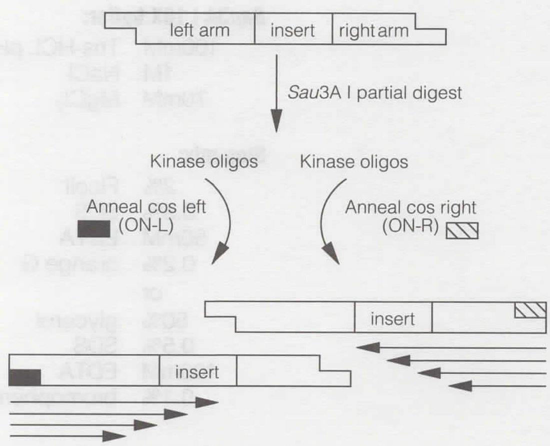

Figure 4. LambdaMap cos site end-labeling system for mapping genomic inserts in lambda cloning vectors.

Reagents to be Supplied by the User

- appropriate restriction enzyme and buffer
- stop buffer (pg. 197)
- [γ-^{32}P]ATP (3,000Ci/mmol)
- T4 polynucleotide kinase
- kinase 10X buffer (pg. 197)
- 100mM DTT

B. Complete Restriction Digestion of Recombinant Lambda DNA

The determination of a restriction map of a recombinant using this technique requires a complete restriction digest of the recombinant lambda DNA using one or more restriction enzymes of the researcher's choice. In order to ensure complete digestion, it is suggested that the researcher double both the manufacturer's recommended enzyme concentration and incubation time.

1. Add 1µg of recombinant lambda DNA to 25µl of the appropriate restriction enzyme 1X buffer.

2. Add double the recommended enzyme concentration. Mix gently. Incubate at the required temperature for double the recommended incubation time. The final volume should be 35µl or less.

3. Divide the samples into 9µl aliquots and add 1µl of stop buffer (pg. 197) to each. Load one sample onto an 0.8% agarose gel. Also, load a sample of standard DNA size markers for gel analysis in the same manner. Run the gel at relatively low voltage to improve linearity of mobility versus size for large fragments.

Notes:

1. Samples of complete restriction digests not used for gel analysis at this stage may be stored at –20°C.

2. Agarose gels may run in the presence of or be poststained with ethidium bromide (0.5µg/ml).

VII. Restriction Mapping of Recombinant Lambda DNA with the LambdaMap™ System

(continued)

C. Partial Restriction Digestion of Recombinant Lambda DNA

1. To generate the most concise pattern of all possible partial restriction fragments, it is necessary to prepare a dilution range of the appropriate restriction enzyme. A convenient range is as follows:

   6.4u/µl 1.6u/µl 0.4u/µl 0.1u/µl

   (If used immediately, the restriction enzyme may be diluted in restriction enzyme 1X buffer.)

2. For each individual restriction enzyme dilution, add 1µg of recombinant lambda DNA to 25µl of restriction enzyme 1X buffer in a microcentrifuge tube.

3. Add 1µl of each restriction enzyme dilution to the individual tubes of recombinant lambda DNA (prepared in Step 2). Mix gently and incubate for 30 minutes at 37°C. The final volume should be 35µl or less.

4. Add 3µl of 0.5M EDTA to each tube and vortex.

5. Remove 9µl aliquots from each reaction for gel analysis and store the remaining digestions at –20°C until ready to proceed with hybridization to ON-L and ON-R sequences. Add 1µl of stop mix to each 9µl aliquot and analyze each for degree of digestion. In addition to partial digest samples, samples of the band pattern obtained from a complete digest of the recombinant DNA (prepared in Section B) and appropriate DNA size markers should also be run on the gel. Note that the partial digest pattern should be significantly more complex than the "complete digest", with more bands of varying intensities. If the partial and complete digests appear similar, make a further dilution and redigest. If no digestion has occurred, add more enzyme and redigest.

D. Labeling the ON-L and ON-R Oligonucleotides

Prior to labeling, set two water baths to 37°C and 100°C.

1. Add the following to a microcentrifuge tube:

ON-L (stock 200ng/µl)	1.0µl
[γ-^{32}P]ATP (3,000Ci/mmole, 10µCi/µl)	2.0µl
kinase 10X buffer (pg. 197)	1.2µl
100mM dithiothreitol (DTT)	0.5µl
T4 polynucleotide kinase (stock 10u/µl)	0.8µl
H_2O	6.5µl
total volume	12.0µl

   Repeat the above procedure with the ON-R.

2. Mix gently and incubate for 60 minutes at 37°C.

3. Heat the sample for 1 minute at 100°C to inactivate the T4 polynucleotide kinase.

4. Add 188µl of kinase 1X buffer to each tube (final concentration of ON-L or ON-R=1ng/µl).

   Note: If not used immediately, labeled oligos can be stored at –20°C for 4 weeks.

Genomic Cloning and Mapping

VII. Restriction Mapping of Recombinant Lambda DNA with the LambdaMap™ System

(continued)

E. Hybridization of ^{32}P-Labeled ON-L and ON-R to Partial Digestion Products

Prior to hybridization, set two water baths to 75°C and 45°C.

1. Add 2µl of 1M NaCl to each individual 20µl partial digest aliquot of recombinant lambda DNA (prepared in Section C, pg. 195) and vortex. Divide the samples by pipeting two 9µl aliquots from each partial digest into separate microcentrifuge tubes.
2. Add 1µl of ^{32}P labeled ON-L (prepared in Section D, Step 3, pg. 195) to 1 aliquot and 1µl of ^{32}P labeled ON-R to the other. Vortex.
3. Incubate for two minutes at 75°C and then for 30 minutes at 45°C.
4. Add 2µl of stop buffer to each individual aliquot. Vortex.

F. Hybridization of ^{32}P-Labeled ON-L and ON-R to Lambda DNA Size Markers

1. Add 3µl (300ng) of lambda marker DNA (pg. 197) into two individual microcentrifuge tubes. Add 0.6µl of 1M NaCl and 1.5µl of H_2O to each tube. Mix.
2. Add 1µl of ^{32}P-labeled ON-L to 1 tube and 1µl of ^{32}P-labeled ON-R to the other tube. Mix.
3. Incubate both for 2 minutes at 75°C and then for 30 minutes at 45°C.
4. Add 1µl of stop buffer to each tube. Mix. Combine the two together immediately before loading the gel.

G. Gel Electrophoresis and Autoradiography

1. Hybridization mixtures are run on a 0.4% agarose gel in Tris-acetate electrophoresis buffer (pg. 197).
2. Load gel apparatus with each recombinant lambda DNA sample.
3. Gels are run at 1.5 volts/cm and require 28-40 hours for optimal separation.
4. After electrophoresis, cut 1 piece of 3MM filter paper slightly larger than the gel and 2 pieces to the size of the gel.
5. Cut 1 piece of nylon transfer membrane to the size of the gel and soak it in H_2O for 20 minutes.
6. Rinse the gel briefly in H_2O and trim the well portion from the top of the gel.
7. Place the oversized piece of 3MM paper in a shallow tray. Place the gel on top of it and the wetted nylon membrane on top of the gel.
8. Briefly soak the two remaining pieces of 3MM filter paper in H_2O and place on top of the nylon.
9. Place 2 or 3 layers of blotting material on top of the gel.
10. Place an evenly distributed 1kg weight on top of the gel and compress the gel for 2 hours.
11. Remove the weight, blotting material, and 3MM filter paper. Wrap the gel and nylon membrane with plastic wrap. Place a piece of XAR-5 film on the wrapped gel and place in an autoradiograph holder for 2 days. If an intensifying screen is used, exposure is complete in 5-7 hours.

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VII. Restriction Mapping of Recombinant Lambda DNA with the LambdaMap™ System

(continued)

H. Interpretation of Results

Each of the radioactively labeled bands in the gel indicates the distance between the radioactive end ([^{32}P]ON-L or [^{32}P]ON-R:cos site duplex) and a site which was digested by the restriction enzyme. Since this data represents a partial digest, not every molecule was digested at every restriction site within the molecule, so different molecules will have different lengths depending on which of the sites was cut. The longest (i.e., slowest migrating) fragment in the gel (the first band down from the gel origin) represents no restriction enzyme digestion. The next band down from the origin represents the restriction site farthest from the labeled end. The distance between adjacent labeled bands in the gel is the distance between adjacent potential cut sites along the recombinant DNA molecule. From the relative positions of bands down the gel, one can distinguish the order of fragments and cut sites along the recombinant DNA molecule and the sizes of fragments which would be produced by a complete digest. The complete digest provides a confirmation of the distance between adjacent restriction sites. Measuring from the opposite end (hybridization with opposite cos site) should confirm the order of fragments and sites.

I. Sizes of Molecular Weight Markers for the LambdaMap System

Restriction digested lambda DNA is supplied with this system as molecular weight markers which are ready to hybridize to the ON-L and ON-R probes. Of the 15 different-sized fragments present, 11 contain cos sites and will be labeled by the oligonucleotide probes. The sizes of these fragments (in base pairs) are listed below.

15 fragments (unlabeled)			11 fragments (labeled)	
48,500	22,600	10,100	48,500	22,600
38,500	19,400	8,600	38,500	19,400
33,500	17,000	8,200	33,500	17,000
29,900	15,000	1,503	29,900	15,000
24,800	12,200	1,105	24,800	10,100
			8,600	

Composition of Solutions

Stop buffer (100ml):

20ml	0.5M EDTA, pH 8.0
80ml	10% glycerol
2.5mg/ml	bromophenol blue
10mg/ml	xylene cyanol

Store at 4°C.

Kinase 10X buffer (10ml):

500mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
50mM	DTT
1mM	spermidine

Tris-acetate electrophoresis buffer:

40mM	Tris-base, pH 7.8
10mM	sodium acetate
1mM	EDTA (disodium)
28mM	acetic acid

Genomic Cloning and Mapping

VIII. References

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IX. Additional Genomic Cloning and Mapping Literature Available from Promega

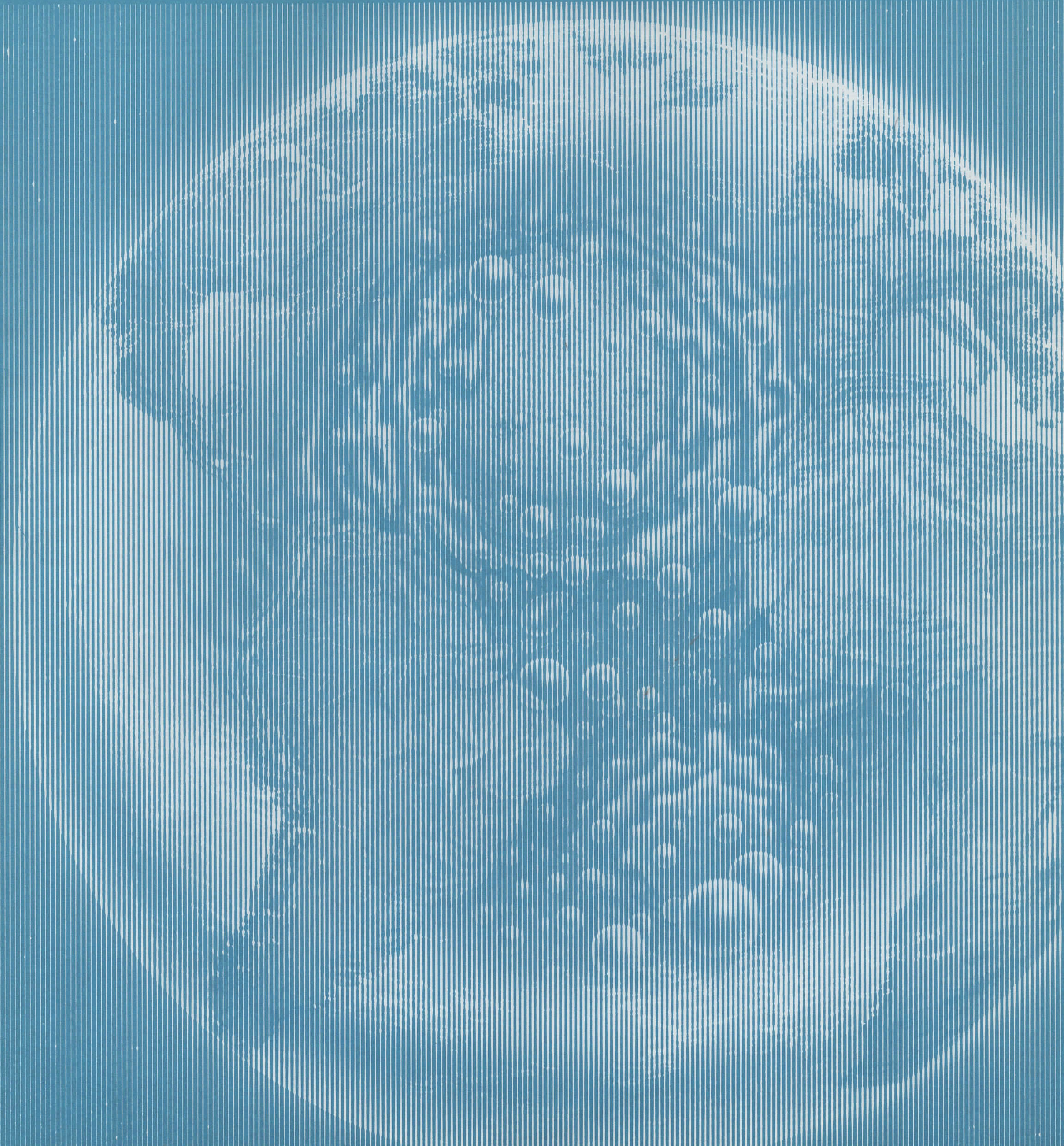
Technical Bulletins

005	Packagene® *in vitro* Packaging System; User Information
037	EMBL3 *Bam*H I Arms: Positive Control Protocol
046	LambdaMap™ System
053	Preparation of LambdaGEM®-11 Arms for Genomic Cloning
055	LambdaGEM®-11 *Bam*H I Half-Site Arms Cloning System
057	LambdaGEM®-11 *Xho* I Half-Site Arms Cloning System
059	Preparation of EMBL Arms for Genomic Cloning
061	Size Fractionation of Genomic DNA
063	*Sfi* Linker Mapping System
065	Preparation of LambdaGEM®-12 Arms for Genomic Cloning
077	LambdaGEM®-12 *Xho* I Half-Site Arms Cloning System

Promega Notes Articles

Issue	Title
6	Rapid isolation of lambda DNA with LambdaSorb® phage adsorbent
11	Efficient genomic library construction: ultra-low background of nonrecombinant with EMBL3-*Bam*H I arms
13	A unique lambda vector engineered for high resolution restriction mapping of genomic inserts: LambdaGEM®-11 vector
14	Efficient construction of unbiased genomic libraries using unfractionated DNA LambdaGEM®-11 *Xho* I half-site arms
18	Gel electrophoresis of lambda DNA:NaCl phenomenon
25	An evaluation of background levels of LambdaGEM®-11 *Xho* I half-site arms
25	Efficiency of packaging lambda DNA *in vitro* in the presence of tRNA

PROTOCOLS AND APPLICATIONS GUIDE



cDNA Synthesis and Cloning

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cDNA Synthesis and Cloning

Contents

(continued)

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cDNA Synthesis and Cloning

I. General Considerations for cDNA Cloning

A. Alternate Strategies for cDNA Cloning

Among the many methods that have been developed in molecular biology, one of the most challenging has been the synthesis and cloning of cDNA. A complex series of enzymatic steps is involved in copying mRNA into double-stranded cDNA and subsequently preparing the termini for vector ligation. Many approaches have been used to generate cDNA libraries, and these have tried to preserve as much of the original sequence as possible, to improve cloning efficiency, and to facilitate screening and subsequent analysis (for a review, see reference 1). The RiboClone® system has been developed to simplify the steps involved in synthesizing double-stranded cDNA using RNA from any source. The method is based on that described by Okayama and Berg (2), as later modified by Gubler and Hoffman (3). First strand synthesis is driven by AMV reverse transcriptase and either random primers or an oligo(dT)-containing primer, followed directly by second strand replacement synthesis using RNase H, DNA polymerase I and *E. coli* DNA ligase. After treatment with T4 DNA polymerase to flush the ends, the double-stranded cDNA molecules are ready for cloning by a variety of methods.

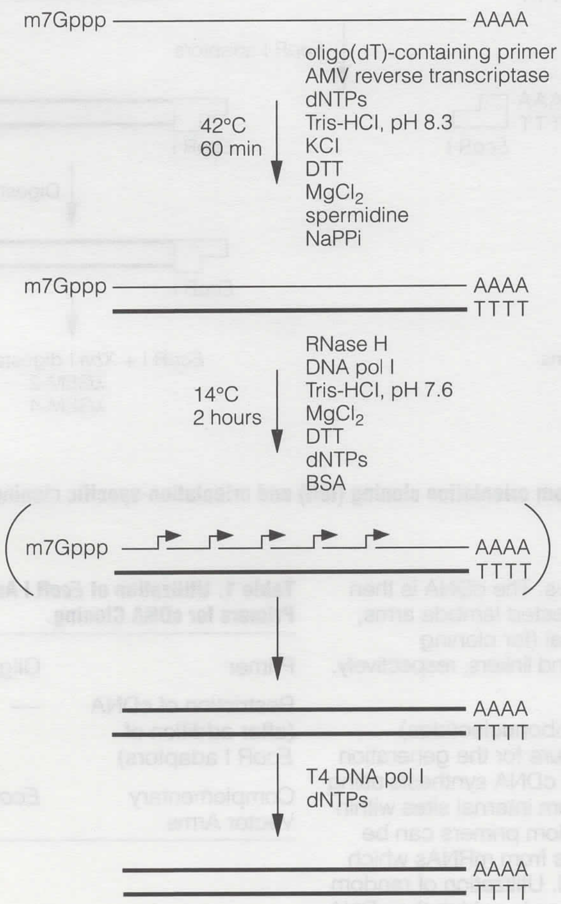


Figure 1. RiboClone system cDNA synthesis scheme using oligo(dT)-containing primers.

Four systems are available, differing only in the primer that is supplied. As shown in Figure 1, the classical oligo(dT) primer can be used when preparing cDNA libraries in vectors like λgt10 and λgt11, where inserts are cloned into a single *Eco*R I site via the addition of *Eco*R I adaptors (or linkers) to each end of the cDNA molecules. However, a more powerful method, which allows efficient orientation-specific cloning of cDNA, is possible using a modified "primer-adaptor" to prime first strand synthesis.

Orientation-specific, or directional, cDNA cloning is valuable in two major applications. In expression vectors such as Lambda gt11 *Sfi-Not*, this method of cDNA cloning can increase by a factor of two the likelihood of expressing the in-sert as the correct polypeptide. In transcription vectors such as the LambdaGEM®-2* and LambdaGEM®-4* vectors, all of the inserts are cloned in the same orientation relative to the promoters for T7 and SP6 RNA polymerase. Total DNA from the resulting library can be transcribed to obtain either sense or antisense RNA probes, respectively, that represent all of the sequences in the library. Because this type of transcription is so efficient and specific, these RNAs can be used to drive subtraction hybridization procedures for the isolation and analysis of rare genes expressed in one tissue but not another (4). (See Section XI, pg. 234 for more details.)

Directional cloning is made possible by using a primer-adaptor in first strand synthesis that consists of oligo(dT) adjacent to a unique restriction site (*Xba* I or *Not* I) (Figure 2. See Table 4, pg. 207 for sequence information). Subsequent procedures are carried out as for oligo(dT)-primed synthesis, except that the final double-stranded cDNA with *Eco*R I adaptors attached is digested with either *Xba* I or *Not* I, resulting in molecules with one *Eco*R I terminus

*U.S. Pat. No. 4,766,072 has been issued to transcription vectors having two different promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

cDNA Synthesis and Cloning

I. General Considerations for cDNA Cloning

(continued)

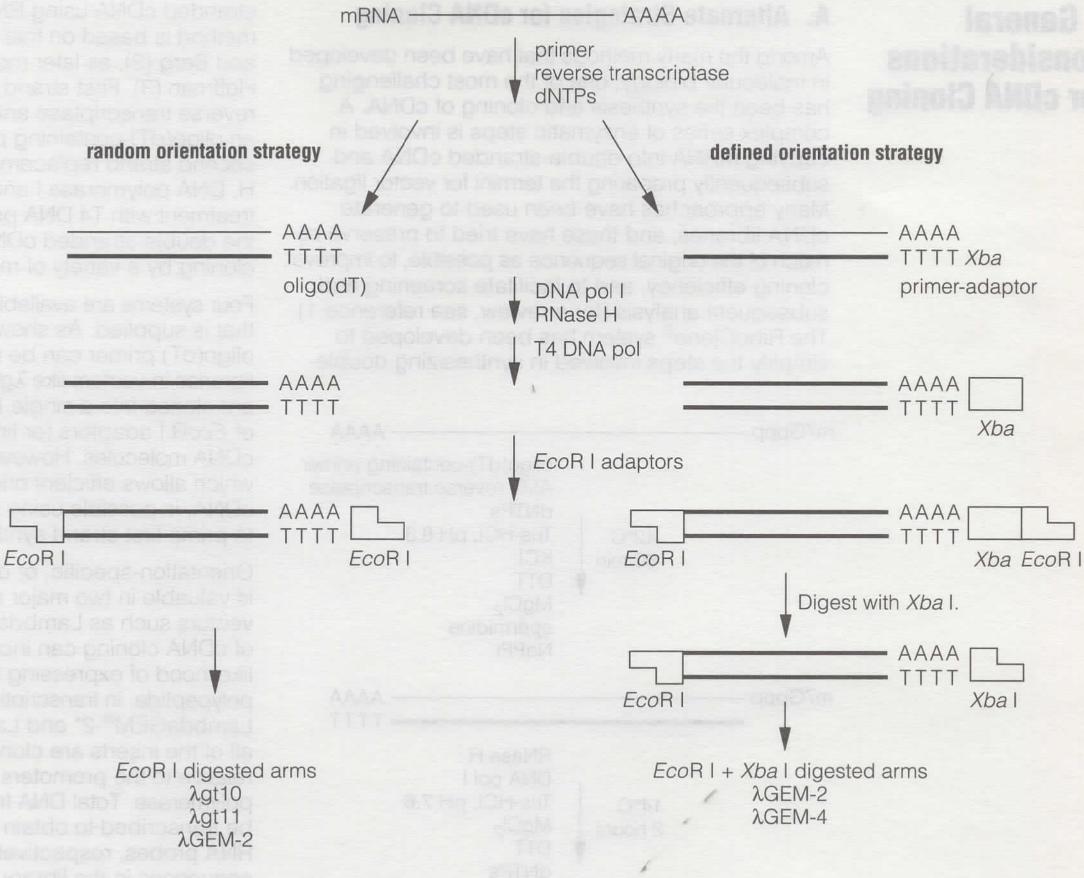


Figure 2. Diagram showing random orientation cloning (left) and orientation-specific cloning (right).

and one *Xba* I or *Not* I terminus. The cDNA is then ligated to the appropriate digested lambda arms, packaged, and plated as usual (for cloning requirements using adaptors and linkers, respectively, see Tables 1 and 2).

Random primers (hexadeoxyribonucleotides) provide an alternative procedure for the generation of cDNA libraries. First strand cDNA synthesis using random primers is initiated from internal sites within the mRNA molecule (5). Random primers can be used to prime cDNA synthesis from mRNAs which do not possess a poly(A)+ tail. Utilization of random primers also provides a scheme by which the cDNA can be synthesized representing the 5′ region of RNA with strong secondary structure (Figure 3).

Table 1. Utilization of *Eco*R I Adaptors and Appropriate Primers for cDNA Cloning.

Primer	Oligo(dT)	*Xba* I	*Not* I
Restriction of cDNA (after addition of *Eco*R I adaptors)	—	*Xba* I	*Not* I
Complementary Vector Arms	*Eco*R I	*Eco*R I *Xba* I	*Eco*R I *Not* I

I. General Considerations for cDNA Cloning

(continued)

Table 2. Utilization of *Eco*R I Linkers and Appropriate Primers for cDNA Cloning.

Primer	Oligo(dT)	*Xba* I	*Not* I
Restriction of cDNA (after methylation of cDNA and addition of *Eco*R I linkers)	*Eco*R I	*Eco*R I *Xba* I	*Eco*R I *Not* I
Complementary Vector Arms	*Eco*R I	*Eco*R I *Xba* I	*Eco*R I *Not* I

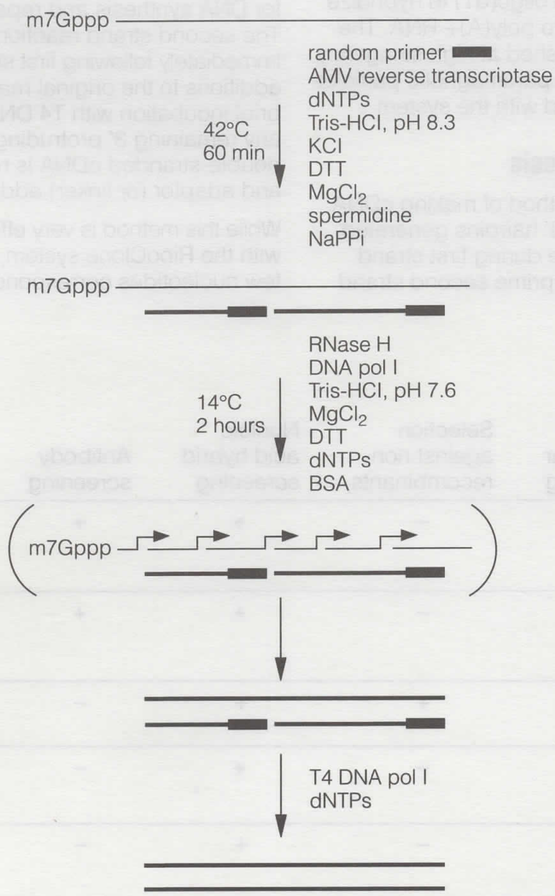


Figure 3. RiboClone system cDNA synthesis scheme using random primers.

B. RNA Preparation and Handling

The quality and quantity of cDNA synthesized by any method is critically dependent on the integrity of the mRNA used as the template. Extremely stable RNases are ubiquitous in the laboratory environment (including fingers of the researcher and, to various extents, in all cells), and therefore certain precautions should be taken to eliminate the risk of RNase contamination whenever possible.

Procedures for creating an RNase-free laboratory environment have been described in detail (6). Sterile disposable plastic test tubes, individually wrapped pipets, micropipet tips and microcentrifuge tubes should be used. Disposable gloves should be worn at all times and changed often. All glassware, non-disposable plasticware and electrophoresis apparatus used for RNA work should be kept separate from other labware. Glassware should be soaked and rinsed in a solution of 0.1% diethyl pyrocarbonate (DEPC), autoclaved and baked at least 3 hours at 250°C before use. Proper health precautions should be taken when using DEPC, which is a powerful acylating agent. This compound should always be used in a fume hood, and never added to aqueous solutions containing ammonia, which results in the formation of ethyl carbamate, a potent carcinogen (7).

Solutions to be used with RNA should be treated with 0.1% DEPC, which inhibits RNase by acylation. This is done by adding the agent, which can be diluted in ethanol if necessary, stirring vigorously for 10 minutes, and then heating the solution to remove the DEPC. Heat treatment can be accomplished by autoclaving or, where indicated, by heating at 70°C for 1 hour with occasional mixing or at 60°C overnight. It is important to note that DEPC will react with amines and sulfhydryl groups, so reagents such as Tris and DTT cannot be treated directly. Tris solutions can be prepared in DEPC-treated water and autoclaved. For reagents, such as DTT, that cannot be autoclaved, solutions should be prepared using DEPC-treated water and then sterile-filtered.

Many procedures have been reported for the isolation of undegraded RNA from various cells and tissues. A widely used method that has been successful with RNase-rich sources was first described by Chirgwin *et al.* (8). Improvements in this method have been reported more recently (9,10) so that sufficient amounts of RNA can be isolated from a number of samples in several hours. Promega's RNAgents™ total RNA isolation system provides a rapid and convenient means to isolate high-quality total RNA (pg. 125).

cDNA Synthesis and Cloning

I. General Considerations for cDNA Cloning

(continued)

The standard method of preparing poly(A)+ RNA has been by chromatography on oligo(dT) cellulose (11). It should be noted that, even after two cycles of oligo(dT) selection, most preparations still contain varying amounts of ribosomal RNA sequences. In general, these sequences will not interfere with cDNA synthesis using oligo(dT)-containing primers. However, it is especially important to eliminate as much ribosomal RNA (or poly(A)– RNA) as possible when constructing cDNA libraries utilizing random primers. See pg. 132 for a method of determining the quality of poly(A)+ RNA preparations. Promega's PolyATtract™ mRNA isolation system (pg. 130) allows mRNA to be isolated from total RNA in under 45 minutes, with only a single round of purification. The system uses biotinylated oligo(dT) to hybridize at high efficiency in solution to poly(A)+ RNA. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles and a magnetic rack included with the system.

C. Method of cDNA Synthesis

A widely used "classical" method of making cDNA (12) takes advantage of the 3′ hairpins generated by AMV reverse transcriptase during first strand synthesis, which are used to prime second strand synthesis catalyzed by Klenow DNA polymerase and reverse transcriptase. After completion of the second strand, S1 nuclease is used to cleave the hairpin loop. This digestion is difficult to control and is the major cause of low cloning efficiencies and the loss of a significant amount of sequence information corresponding to the 5′ end of the mRNA (1,3).

A substantial improvement in cDNA synthesis has been made that avoids the use of S1 nuclease (2,3). In this method, first strand synthesis is performed in the presence of 4mM sodium pyrophosphate, which greatly suppresses the formation of hairpins (1). Second strand synthesis is then carried out using RNase H to create nicks and gaps in the hybridized mRNA strand, which provides 3′-OH priming sites for DNA synthesis and repair by DNA polymerase I. The second strand reaction can be carried out immediately following first strand synthesis by simple additions to the original reaction mixture. After a brief incubation with T4 DNA polymerase to remove any remaining 3′ protruding ends, the blunt-ended, double-stranded cDNA is ready for fractionation and adaptor (or linker) addition.

While this method is very efficient and easy to perform with the RiboClone system, it should be noted that a few nucleotides corresponding to the 5′ end of the

Table 3. Properties of Lambda cDNA Cloning Vectors.

Vector	Cloning capacity	Directional cloning	Blue/clear screening	Selection against non-recombinants	Nucleic acid hybrid. screening	Antibody screening	SP6/T7 promoters	Strains provided
λgt11	0-7.2kb		+	–	+	+	–	Y1090 Y1089 LE392
λgt11 *Sfi-Not*	0-7.2kb	+ *Not* I-*Eco*R I	+	–	+	+	–	Y1090 Y1089 LE392
λgt10	0-7.6kb	–	–	+	+	–	–	C600 C600*hfl*
λGEM-2	0-7.1kb	+ *Xba* I-*Eco*R I		–	+	–	+	LE392
λGEM-4	0-4.7kb	+ *Xba* I-*Eco*R I		–	+	–	+	LE392

I. General Considerations for cDNA Cloning

(continued)

mRNA will be lost during second strand synthesis. This is because second strand replacement can only proceed from 3′-OH RNA primers randomly generated by the combined activities of both the added RNase H and the endogenous RNase H activity of reverse transcriptase. The 5′-most nick in the mRNA will probably occur several nucleotides from the end, and the remaining RNA oligonucleotide may be too short to remain hybridized. Thus, the 3′→5′ exonuclease activity of DNA polymerase I will remove the very last few nucleotides of the cDNA first strand. However, since all eukaryotic mRNAs appear to have 5′ non-coding leader sequences, which commonly range from 40 to 80 nucleotides (13), it is likely that the vast majority of double-stranded cDNA will contain all of the coding sequences present in the initial cellular mRNAs.

If it is necessary to retain these nucleotides, alternative procedures involving tailing after first strand synthesis are possible (1,9). Homopolymeric tailing is best accomplished by adding dG residues (14), and the resulting clones will contain a long stretch (about 20bp) of dG/dC residues. These regions may interfere with screening using GC-rich oligomers, with dideoxy sequencing of clones, and possibly with their expression as part of a fusion protein (14). In addition, these regions can interfere with differential hybridization procedures in the construction of subtraction libraries (pg. 234).

D. Vectors

The choice of vector for cDNA cloning depends on the method which will be used for screening, on the ease with which the resulting clones can be grown and manipulated, and on the number of recombinants necessary to ensure the presence of the desired sequence. For example, about 2 x 10^5 recombinants must be screened (using nucleic acid probes) to detect a low abundance mRNA from mammalian cells (15). In choosing between plasmid and bacteriophage lambda vectors, several factors must be considered. In general, lambda vectors are preferred when large numbers of recombinants are needed. This is because high cloning efficiencies (10^7pfu/µg arms) are consistently obtained via *in vitro* packaging reactions and subsequent infection. In addition, it is possible to efficiently screen large numbers of recombinants at high plaque densities with either nucleic acid or antibody probes (16-19). Table 3 lists the properties of a number of lambda cDNA cloning vectors. With plasmids, cloning efficiencies are usually 10- to 50-fold lower, colonies must be screened at much lower densities, and screening is

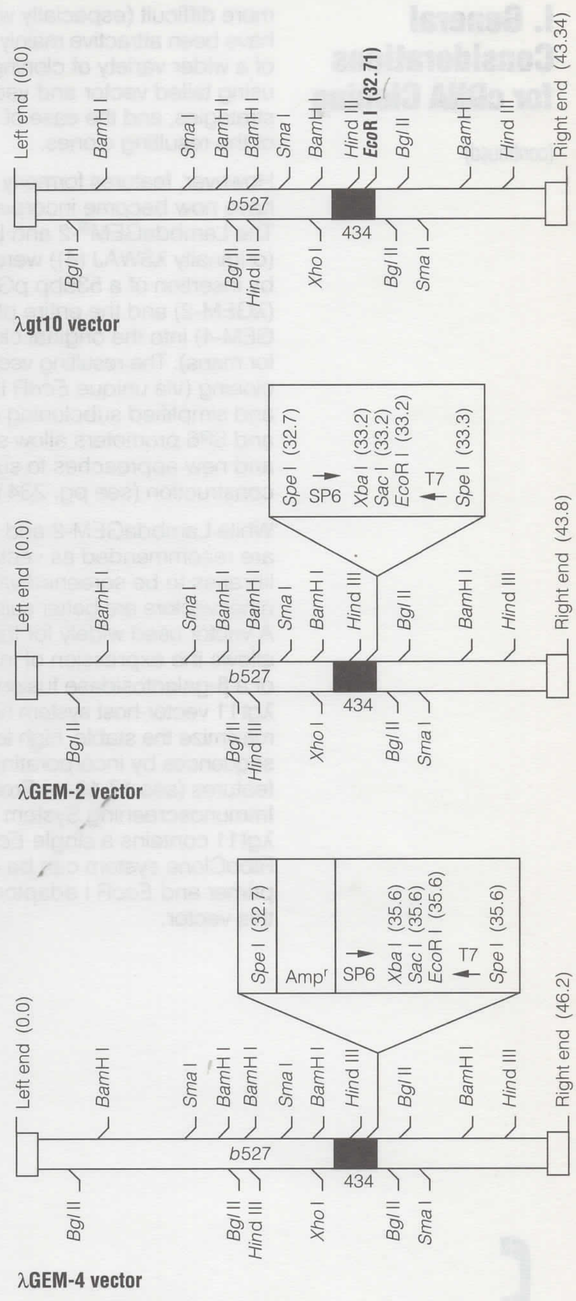


Figure 4. Maps of λgt10, λGEM-2 and λGEM-4 cDNA cloning vectors.

cDNA Synthesis and Cloning

I. General Considerations for cDNA Cloning

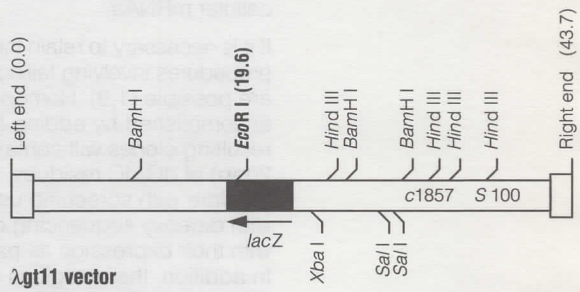
(continued)

more difficult (especially with antibodies). Plasmids have been attractive mainly because of the availability of a wider variety of cloning sites, possibilities for using tailed vector and vector-primer cloning strategies, and the ease of isolation and manipulation of the resulting clones.

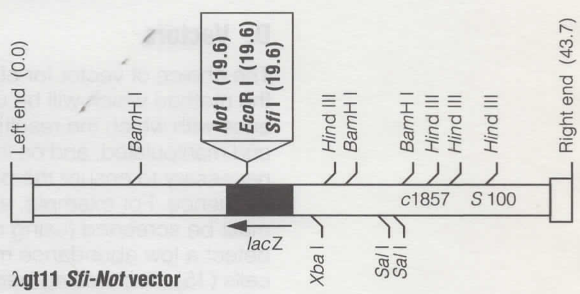
However, features formerly available only in plasmids have now become incorporated into phage vectors. The LambdaGEM®-2 and LambdaGEM®-4 vectors (originally λSWAJ (4)) were constructed from λgt10 by insertion of a 535bp pGEM®-1 vector fragment (λGEM-2) and the entire pGEM-1 plasmid (LambdaGEM-4) into the original cloning site (see Figure 4 for maps). The resulting vectors incorporate directional cloning (via unique *Eco*R I and *Xba* I cloning sites), and simplified subcloning capabilities, and the T7 and SP6 promoters allow simplified probe synthesis and new approaches to subtraction library construction (see pg. 234 for details).

While LambdaGEM-2 and LambdaGEM-4 vectors are recommended as vectors for constructing libraries to be screened with nucleic acid probes, other vectors are better suited for antibody screening. A vector used widely for this purpose is λgt11, which allows the expression of inserted sequences as part of a β-galactosidase fusion protein (17-19). The λgt11 vector-host system has been constructed to maximize the stable, high level expression of foreign sequences by incorporating a number of important features (see 17,19, or Promega's ProtoBlot® Immunoscreening System Manual for details). Since λgt11 contains a single *Eco*R I cloning site, the RiboClone system can be used with oligo(dT) as primer and *Eco*R I adaptors or linkers for cloning in this vector.

Another vector is available which features all the advantages of λgt11 and, in addition, allows directional cloning of cDNA. The λgt11 *Sfi-Not* vector is based upon λgt11 and features unique *Sfi* I, *Eco*R I, and *Not* I cloning sites. This vector can be used in the same directional cloning scheme as for the λGEM-2 and λGEM-4 vectors, thus doubling the likelihood of in-frame expression of inserts as fusion polypeptides. This cloning strategy effectively halves the number of recombinants that need to be screened to detect a particular expressed sequence. The bacterial strains used with each vector are listed in Table 3 (pg. 204) and are described more fully in the Technical Appendix (pg. 405).



λgt11 vector



λgt11 *Sfi-Not* vector

Figure 5. Maps of λgt11 and λgt11 *Sfi-Not* cDNA cloning vectors.

I. General Considerations for cDNA Cloning

(continued)

E. Screening cDNA Libraries

Two approaches are commonly used for screening cDNA libraries. Immunoscreening of an expression library in the λgt11 or λgt11 *Sfi-Not* vectors is convenient and effective. This approach is described in Section VI, pg. 221. Immunoscreening requires that an antibody to the target protein be available and that the antibody not crossreact with the host proteins. Sometimes, the limited amount of protein which can be purified by SDS-polyacrylamide gel electrophoresis does not easily allow such an antibody to be produced.

A common alternative is to use nucleic acid probes to screen cDNA libraries in the λgt10, λGEM-2 or λGEM-4 vectors as well as in the other vectors listed above. To design such a probe, a small protein of the protein sequence encoded by the gene of interest must be known. Protein sequence information can be obtained using small amounts of protein (2-25μg), but the protein must first be prepared for sequencing, as described on pgs. 256-261.

Table 4. Oligonucleotides and Primers for use in cDNA Synthesis and Cloning.

Oligonucleotide	Function	Sequence
Xba I Primer-Adaptor	Primer for first strand cDNA synthesis in RiboClone cDNA synthesis system	5′-GTCGACTCTAGA(T)$_{15}$-3′
Not I Primer-Adaptor	Primer for first strand cDNA synthesis in RiboClone cDNA synthesis system	5′-CAATTCGCGGCCGC(T)$_{15}$-3′
Oligo(dT)$_{15}$ Primer	Primer for first strand cDNA synthesis	5′-(T)$_{15}$-3′
T7 Spacer Primer	Used in the construction of subtraction libraries using the LambdaGEM-2 or LambdaGEM-4 vectors	5′-GGGAGACCGGAATTC-3′
Xba I-*Eco*R I Polylinker Oligo	Used for the screening of nonrecombinants in the LambdaGEM-2 or LambdaGEM-4 vectors	5′-GAATTCGAGCTCGCCCGGGGATCCTCTAGA-3′
*Eco*R I Adaptors	Used to generate *Eco*R I sites at the termini of blunt-ended cDNA. No need to digest with *Eco*R I.	5′-AATTCCGTTGCTGTCG-3′ 3′-GGCAACGACAGCp-5′
*Eco*R I Linkers	Used to generate *Eco*R I sites at the termini of blunt-ended cDNA. Digestion with *Eco*R I endonuclease required.	5′-pCGGAATTCCG-3′ 3′-GCCTTAAGGCp-5′

Notes: The T7 spacer primer corresponds to the sequence between the initiation of T7 RNA polymerase transcription and the *Eco*R I cloning site in LambdaGEM-2 and LambdaGEM-4 vectors. This oligonucleotide is used to prime second strand cDNA synthesis in the construction of subtraction libraries with these vectors (see pg. 234). The *Eco*R I-*Xba* I polylinker oligo corresponds to the sequence from the *Eco*R I site to the *Xba* I site in these vectors. It can be conveniently labeled using [γ-^{32}P]ATP and polynucleotide kinase and used to screen for nonrecombinants, since this sequence is absent in orientation-specific clones containing inserts.

cDNA Synthesis and Cloning

II. cDNA Synthesis Using the RiboClone® System

A. First Strand Synthesis

The standard reaction described below is set up in a total volume of 25µl, which is recommended for up to 2µg of mRNA. For each additional microgram of mRNA, increase the reaction volume by 10µl. For example, the reaction should be scaled up to 55µl when using 5µg of mRNA. After all of the components are added, a 5µl sample is removed to a separate tube containing a small amount of [α-^{32}P]dCTP as a tracer to follow first strand synthesis. The remainder of the reaction is carried through second strand synthesis.

Ratios of primer or primer-adaptor and reverse transcriptase to mRNA should be maintained at 0.5µg/µg (0.3µg/µg for the *Not* I primer-adaptor) and 15u/µg, respectively.

Reagents to be Supplied by the User

- [α-^{32}P]dCTP (>400Ci/mmol)
- EDTA (50mM and 200mM)
- TE-saturated phenol/chloroform (pg. 213)
- 7.5M ammonium acetate
- ethanol (100% and 70%)
- TE buffer (pg. 213)

The reaction below is designed for using 1µg of the 1.2kb positive control RNA.

1. In a sterile RNase-free microcentrifuge tube, add the primer or primer-adaptor to the mRNA sample. Use 0.5µg primer/µg mRNA (0.3µg/µg mRNA for the *Not* I primer-adaptor) in a total volume of up to 15µl in water and heat to 70°C for 5 minutes. Let cool to room temperature, spin the tube briefly to collect the solution at the bottom, and add the following components to the annealed primer/template in the order shown. Before addition of sodium pyrophosphate and AMV reverse transcriptase, preheat the reaction components and sodium pyrophosphate to 42°C for 5 minutes. This heating step prevents precipitation of the sodium pyrophosphate when it is added to the buffer components.

first strand 5X buffer (pg. 213)	5µl
rRNasin® ribonuclease inhibitor	25u
40mM sodium pyrophosphate	2.5µl
AMV reverse transcriptase	15u/µg RNA
nuclease-free water	to final volume 25µl

2. Mix gently by flicking the tube, and remove 5µl of the mixture to another tube containing 2-5µCi of [α-^{32}P]dCTP (>400Ci/mmol), which has either been dried down or is in less than 1µl volume. This is the tracer reaction, which will be used to measure first strand synthesis by incorporation (TCA precipitation) and alkaline agarose gel electrophoresis (pg. 211).

Note: We recommend using [α-^{32}P]dCTP (aqueous form) of specific activity >400Ci/mmol, such as Amersham Cat. No. PB10165, and which is less than 1 week old.

3. Incubate both reactions at 42°C for 60 minutes.

4. Place both tubes on ice.

5. Add 50mM EDTA to the tracer reaction up to a total volume of 100µl and store on ice. Save 10µl of the reaction for incorporation assays (pg. 210). The remaining 90µl can be used for gel analysis (pg. 211) after extraction.

6. Move on to second strand synthesis using the larger, unlabeled first strand reaction.

The final reaction conditions for first strand synthesis are:

50mM	Tris-HCl, pH 8.3
50mM	KCl
10mM	$MgCl_2$
0.5mM	spermidine
10mM	DTT
4mM	sodium pyrophosphate
1mM each	dATP, dCTP, dGTP, dTTP
1u	rRNasin® ribonuclease inhibitor/µl reaction
0.3-0.5µg	primer/µg RNA
15u	AMV reverse transcriptase/µg RNA

B. Second Strand Synthesis

The second strand reaction is performed directly following the first strand reaction. No extractions or precipitations are necessary.

The second strand reaction volume is based on that of the first strand reaction. A 5-fold dilution of the first strand reaction should be maintained, so the component amounts are scaled proportionately to those listed below for the 20µl first strand volume.

II. cDNA Synthesis Using the RiboClone® System

(continued)

1. Add the following components to the first strand tube on ice in the order shown:

Component	Amount
first strand reaction	20µl
second strand 10X buffer (pg. 213)	10µl
E. coli DNA polymerase I	23 units
E. coli RNase H	0.8 units
nuclease-free water	to final volume 100µl

2. Mix gently. If a tracer reaction for the second strand is desired, remove 10µl to another tube containing 2-5µCi [α-^{32}P]dCTP. The [α-^{32}P] can be added directly to the 100µl reaction derived from the 1.2kb positive control RNA.

3. Incubate the reactions at 14°C for 2 hours.

   Note: When it is important to synthesize cDNA longer than 3kb, increase the second strand incubation time to 3-4 hours.

4. Add 90µl of 50mM EDTA to the 10µl tracer reaction. Add 10µl of 200mM EDTA to the 100µl control RNA tracer reaction. The tracer reactions can be used for incorporation assays (pg. 210) and gel analysis (pg. 211). Store on ice.

5. Heat the primary, unlabeled sample to 70°C for 10 minutes, collect the contents at the bottom of the tube by brief centrifugation and place on ice.

6. Add 2 units of T4 DNA polymerase/µg input RNA to the primary reaction. Incubate at 37°C for 10 minutes.

7. Stop the reaction by adding 10µl of 200mM EDTA and place on ice.

8. To extract the cDNA, add an equal volume of TE-saturated phenol/chloroform (pg. 213), mix thoroughly, and centrifuge in a microcentrifuge for 2 minutes at room temperature.

   Note: It is often convenient to extract the tracer reactions along with the primary second strand reaction. After saving 10µl of the tracer reactions for incorporation assays, extract with TE-saturated phenol/chloroform and precipitate with ethanol as described in Steps 7-10.

9. Transfer the aqueous phase to a fresh tube, add 0.5 volume of 7.5M ammonium acetate (or 0.1 volume of 2.5M sodium acetate, pH 5.2), mix, and add 2.5 volumes of cold (–20°C) ethanol. Cool for 30 minutes at –20°C and centrifuge for 5 minutes.

10. Carefully remove the supernatant, add 0.5ml of cold 70% ethanol, and spin again for 2 minutes.

11. Carefully remove the supernatant and dry the pellet.

12. Dissolve the pellet in 10-50µl of TE buffer. The cDNA is now ready for gel analysis (pg. 211) and size fractionation prior to adaptor or linker addition. Dissolve the tracer reactions in 10-20µl of TE buffer.

The final reaction conditions for second strand synthesis are as follows:

50mM	Tris-HCl, pH 7.6
100mM	KCl
5mM	$MgCl_2$
50µg/ml	bovine serum albumin
5mM	DTT
8u/ml	RNase H
230u/ml	*E. coli* DNA polymerase I

from first strand reaction:

0.2mM each	dATP, dCTP, dGTP, dTTP
0.1mM	spermidine
0.8mM	sodium pyrophosphate

Reagents to be Supplied by the User

- 1mg/ml carrier DNA (e.g., salmon sperm)
- trichloroacetic acid (5% and 7%)
- alkaline agarose gel (21)
- alkaline gel running buffer (pg. 213)
- sample 2X buffer (pg. 213)

cDNA Synthesis and Cloning

II. cDNA Synthesis Using the RiboClone® System

(continued)

C. Incorporation Assay (TCA Precipitation)

1. Spot 3µl samples of the diluted first strand tracer reaction (Step A.5, pg. 208) and the second strand reaction (Step B.4, pg. 209) on glass fiber filters and let air dry. These samples represent the total cpm in the reactions.

2. Add 3µl samples of the same reactions to tubes containing 100µl of a 1mg/ml solution of carrier DNA and mix. Add 0.5ml of 5% trichloroacetic acid (TCA) and vortex. Let sit on ice for 5-30 minutes.

3. Filter the samples through glass fiber filters, washing 3 times with 5ml cold 5% TCA. Rinse the filters with 5ml of acetone or ethanol and let air dry.

4. Count both total and incorporated (TCA precipitable) cpm samples, either by Cerenkov radiation (without scintillant) or after adding an appropriate scintillation fluid.

Note: TCA assays of isotope incorporation are prone to variation (20), and thus some variability can be expected in assays of first and second strand conversion. In our experience, successful cDNA libraries can be constructed from reactions yielding 10-30% first strand conversion and 50-200% second strand conversion values.

D. First Strand Yield Calculation

The first strand yield is determined as follows:

$$\frac{\text{incorporated cpm} \times 100\%}{\text{total cpm}} = \text{\% incorporation}$$

$$4\text{nmoles dNTP/µl} \times \text{reaction vol.(µl)} \times \text{\% incorp.}/100 = \text{nmoles dNTP incorporated}$$

$$\text{nmoles dNTP incorporated} \times 330\text{ng/nmole} = \text{ng cDNA synthesized}$$

$$\frac{\text{ng cDNA synthesized}}{\text{ng mRNA in reaction}} \times 100\% = \text{\% mRNA converted to cDNA}$$

For example, if there were 1,520 cpm incorporated and 254,000 total cpm in the tracer reaction, and 1µg RNA was added to the original 25µl reaction:

$$\frac{1{,}520}{254{,}000} \times 100\% = 0.6\% \text{ incorporation}$$

$$4\text{nmoles dNTP/µl} \times 25\text{µl} \times 0.6/100 = 0.6\text{nmoles dNTP incorporated}$$

$$0.6\text{nmoles dNTP} \times 330\text{ng/nmole} = 198\text{ng cDNA synthesized}$$

$$\frac{198\text{ng cDNA synthesized} \times 100\%}{1{,}000\text{ng mRNA in reaction}} = 19.8\% \text{ mRNA converted to cDNA}$$

Of the original 1,000ng of mRNA, 20% (or 5µl/25µl) was removed for the tracer reaction and 80% (or 20µl/25µl) remained in the main reaction. Therefore, (0.8 x 198ng) or 158ng first strand cDNA were synthesized and (0.8 x 0.6nmoles) or 0.48nmoles dNTP were incorporated in the reaction that was carried through to second strand synthesis.

E. Second Strand Yield Calculation

The second strand yield is calculated in the same manner as the first strand yield, except that the total dNTP in the reaction will be diminished by that which was incorporated during first strand synthesis.

$$\frac{\text{incorporated cpm}}{\text{total cpm}} \times 100\% = \text{\% second strand incorporation}$$

$$[0.8\text{nmoles dNTP/µl} \times \text{reaction volume(µl)} - \text{nmoles incorporated in first strand reaction}] \times \text{\% second strand incorporation}/100 = \text{nmoles dNTP incorporated}$$

$$\text{nmoles dNTP incorporated} \times 330\text{ng/nmole} = \text{ng second strand cDNA synthesized}$$

$$\frac{\text{ng second strand cDNA synthesized} \times 100\%}{\text{ng first strand cDNA synthesized}} = \text{\% conversion to double-stranded cDNA}$$

II. cDNA Synthesis Using the RiboClone® System

(continued)

For example, if the above first strand reaction was used for second strand synthesis, which resulted in 1,390 cpm incorporated out of a total of 235,000 cpm:

$$\frac{1,390}{235,000} \times 100\% = 0.59\% \text{ second strand incorporation}$$

$$[(0.8\text{nmoles}/\mu\text{l} \times 100\mu\text{l}) - 0.48\text{nmoles first strand dNTP incorporated}] \times 0.59/100 = 0.47\text{nmoles dNTP incorporated}$$

$$0.47\text{nmoles} \times 330\text{ng/nmole} = 155\text{ng second strand cDNA synthesized}$$

$$\frac{155\text{ng second strand cDNA}}{158\text{ng first strand cDNA}} \times 100\% = 98\% \text{ conversion to double-stranded cDNA}$$

F. Gel Analysis of cDNA

The size distribution of cDNA synthesized in the first and second strand reactions should be checked by electrophoresis on a 1.4% alkaline agarose gel (15). The samples used for gel analysis are the first strand and second strand tracer reactions that have been extracted with phenol and precipitated with ethanol as described in Section B, Steps 8-12 (pg. 209). It is helpful to load approximately equivalent numbers of incorporated cpm for each strand so that comparisons can be made on the same autoradiograph. As a guideline, about 50,000cpm in one band on a fixed and dried gel results in a dark, sharp signal in a 1 hour exposure with intensifying screens.

1. ^{32}P-labeled DNA markers are convenient for estimating the sizes of the first and second strand products. A convenient way to make these is to use lambda *Hind* III fragments (Promega #G1711) in a fill-in reaction with Klenow DNA polymerase (Promega #M2201). Assemble the following reaction:

Hind III 10X buffer	2.5µl
dATP	0.2mM
dGTP	0.2mM
[α-^{32}P]dCTP	2µCi
lambda *Hind* III markers	1µg
Klenow DNA polymerase	1u
deionized water	to final volume 25µl

Incubate the reaction for 10 minutes at room temperature. Add 2.5µl of 200mM EDTA to stop the reaction. Add a sample directly to sample 2X buffer (pg. 213) and store the remainder at –20°C. A 5µl sample routinely gives a sharp ladder of bands after a 1 hour exposure.

2. Pour a 1.4% alkaline agarose gel in 50mM NaCl, 1mM EDTA and equilibrate for at least 30 minutes in alkaline gel running buffer (pg. 213).

3. Transfer the desired amount of each sample (usually 10,000-50,000 incorporated cpm) to separate tubes, adjust the volumes with TE buffer so that they are the same, and add an equal volume of sample 2X buffer to each.

4. Load the samples and run the gel at up to 7.5V/cm for the desired time (usually until the dye has migrated about 2/3 of the way across the gel).

5. Soak the gel in several volumes of 7% TCA at room temperature for 30 minutes or until the dye changes from blue to yellow and then dry on a piece of Whatman 3MM paper, either on a gel dryer or by placing the gel under a weighted stack of paper towels for several hours.

6. Cover the dried gel with plastic wrap and expose to X-ray film at room temperature or at –70°C with an intensifying screen.

7. If the 1.2kb positive control mRNA was used with an oligo(dT)-containing primer, the first and second strand reaction products should appear as sharp bands at this position in the gel. Random priming results in products which are 1.2kb and smaller.

Note: Denatured DNA samples can also be separated in neutral agarose gels. This convenient alternative to alkaline agarose gels was recently applied to cDNA analysis in a report by Lenstra, *et al.* (21). Samples of DNA to be analyzed are denatured in 0.1N NaOH for 15 minutes prior to loading and electrophoresis in neutral agarose gels. No post-neutralization in TCA or fixing is required before drying the gel in preparation for autoradiography. The agarose gel and running buffer systems can be prepared with the commonly used TBE or TAE buffers. (TBE = 0.89M Tris-base, 0.89M boric acid, 20mM EDTA. TAE = 40mM Tris acetate, pH 7.8, 0.2mM EDTA.)

cDNA Synthesis and Cloning

II. cDNA Synthesis Using the RiboClone® System

(continued)

For successful migration of denatured DNA in the neutral agarose gel system, it is important to begin electrophoresis as soon as possible after loading the denatured samples into the wells.

G. Troubleshooting

Perhaps the most important factor for successful cDNA synthesis using the methods described here is the quality of the mRNA template. The procedures outlined in Section I.B (pg. 203) should be followed to ensure the isolation of undegraded poly(A)+ RNA. Typical results using standard conditions and an oligo(dT) primer or primer-adaptor with the 1.2kb RiboClone system positive control RNA should be about 15% first strand conversion, greater than 80% second strand conversion, and clear bands of the appropriate size as seen on alkaline agarose gels. The second strand may exhibit a small amount of material (less than 5%) as a 2.4kb band, which is the result of hairpin-primed second strand synthesis. In addition, there is usually a faint smear of low molecular weight material (less than 500 bases) in this lane that is probably the result of aberrant priming (1). If these normal results are observed with the positive control, while low incorporation and a large amount of low molecular weight material is seen for the sample RNA, it is likely that there is a problem with the RNA preparation. The following methods can be used to check the integrity of the RNA as it is purified.

It is relatively simple to analyze an RNA preparation by either direct electrophoresis under denaturing conditions [formaldehyde gels are probably the easiest, although methyl mercuric hydroxide gels or glyoxal treatment can also be used (11)] or by analysis of labeled first strand cDNA. If total RNA (not oligo(dT) selected) is run on a gel, bands corresponding to 28S and 18S ribosomal RNA should be apparent, and 28S RNA should stain with approximately twice the intensity of 18S RNA. For cDNA analysis, perform first strand synthesis using 1µg of poly(A)+ RNA with the RiboClone system and run the reaction products on an alkaline agarose gel (pg. 213). A good RNA preparation from almost any tissue will yield cDNA transcripts from 350 to 6,000 bases long and averaging 1,300-1,600 bases. In addition, this reaction can be "spiked" with a small amount of positive control RNA to check for the presence of inhibitors such as SDS, EDTA or salts, which may affect first strand synthesis.

It may be helpful to add an RNA of defined size to the original cell lysate to monitor whether breakdown is occurring during purification. Synthetic RNA of virtually any size can be easily prepared from appropriately linearized recombinants in Riboprobe® Gemini system vectors by transcription *in vitro* using SP6 or T7 RNA polymerase. If it is desired to follow purification through the selection of poly(A)+ molecules, transcripts can be prepared from recombinants in Promega's pSP64(polyA) plasmid, which yields polyadenylated molecules when plasmids are linearized with *Eco*R I. If labeled, this RNA can be detected by autoradiography after electrophoresis on formaldehyde-containing agarose gels (15,22). If this RNA is unlabeled, its integrity may be monitored by probing a northern blot (15,23) of such a gel with labeled DNA or with labeled antisense RNA prepared by transcribing a pGEM® recombinant with the opposing polymerase.

If either antibodies or nucleic acid probes are available, then a target molecule may be followed through the RNA preparation directly either by translation *in vitro* and subsequent immunoprecipitation or by northern analysis. If low incorporation is obtained even with the positive control, it is possible that there is a problem with the batch of radioisotope used as label. It is not necessary to use [α-^{32}P]dCTP; [α-^{32}P]dGTP, as well as other isotopes, can be used as long as appropriate corrections are made for specific activity and counting efficiencies.

Each reaction parameter in the system has been optimized relative to the other components. It is important to follow the protocols carefully with respect to reverse transcriptase:primer:RNA ratios and reaction volumes.

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II. cDNA Synthesis Using the RiboClone® System
(continued)

Composition of Solutions

First strand 5X buffer:

250mM	Tris-HCl, pH 8.3 (at 42°C)
250mM	KCl
50mM	$MgCl_2$
2.5mM	spermidine
50mM	DTT
5mM each	dATP, dCTP, dGTP, dTTP

Second strand 10X buffer:

500mM	Tris-HCl, pH 7.2
900mM	KCl
30mM	$MgCl_2$
30mM	DTT
0.5mg/ml	bovine serum albumin

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:
Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Alkaline gel running buffer:

30mM	NaOH
1mM	EDTA

Make up fresh for each use.

Sample 2X buffer:

20mM	NaOH
20%	glycerol
0.025%	bromophenol blue (added fresh for each use)

*Buffer tablets available from Promega.

III. Ligation of *Eco*R I Linkers to cDNA

The *Eco*R I linker ligation system is designed for the efficient addition of *Eco*R I sites to the termini of blunt-ended DNA molecules (15,24). This system allows cDNA to be cloned into unique *Eco*R I sites of vectors such as λgt10 or λgt11. In addition, cDNA synthesized with an *Xba* I or *Not* I primer-adaptor can be directionally cloned into the *Eco*R I-*Xba* I arms of the LambdaGEM®-2 and LambdaGEM®-4 vectors, or into the *Eco*R I-*Not* I arms of the λgt11 *Sfi-Not* vector (see Figure 2, pg. 202).

Reagents to be Supplied by the User

- TE buffer
- TE-saturated phenol/chloroform
- chloroform:isoamyl alcohol (24:1)
- 2M NaCl
- ethanol (100% and 70%)
- 0.2M EDTA
- 7.5M ammonium acetate

A. Methylation of cDNA

1. cDNA cloning strategies require that the internal *Eco*R I sites of the DNA be protected by *Eco*R I methylation prior to the linker ligation step. The conditions for *Eco*R I methylation are listed below.

1mM S-adenosyl-L-methionine (made by diluting 10mM stock solution with H_2O)	1µl
*Eco*R I methylase 10X buffer (pg. 215)	1µl
BSA (1mg/ml)	1µl
DNA (1mg/ml in H_2O or TE buffer)	1µl
*Eco*R I methylase (per µg DNA)	10u
H_2O	to final volume 10µl

2. Incubate at 37°C for 15 minutes.

3. Heat inactivate the enzyme for 10 minutes at 70°C.

4. Extract with 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and centrifuge at 12,000 x g for 3 minutes.

5. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 minute and centrifuge as in Step 4.

6. Transfer the upper, aqueous phase to a fresh tube. Add 0.1 volume of 2M NaCl. Add 2 volumes of ethanol and leave at –70°C for 30 minutes. Centrifuge at 12,000 x g for 15 minutes.

7. Carefully pour off the supernatant, wash the pellet with 1ml 70% ethanol, dry briefly in a vacuum desiccator, and resuspend in TE buffer at approximately 100ng/µl.

cDNA Synthesis and Cloning

III. Ligation of *Eco*R I Linkers to cDNA

(continued)

B. Ligation of Linkers to cDNA

1. To add *Eco*R I phosphorylated linkers to a DNA fragment, set up the following reaction (see Note 1, pg. 215). The volumes can be scaled up proportionately, depending upon the amount of DNA.

ligase 10X buffer (pg. 215)	1µl
bovine serum albumin (1mg/ml)	1µl
DNA (100ng/µl)	2µl
T4 DNA ligase (Weiss units)	2.5u
*Eco*R I linkers (Dilution of stock linkers can be done in H_2O.)	50-fold molar excess
H_2O	to final volume 10µl

***Example:** 200ng of a 3kb DNA = 0.2pmole ends

0.001 A_{260} units of phosphorylated *Eco*R I linkers = 10pmole

2. Incubate at 15°C for 6-18 hours.
3. Heat inactivate the reaction at 70°C for 10 minutes (see Note 2, pg. 215).

C. Digestion with *Eco*R I

1. Directly after inactivating the ligase (Step B.3), cool the tube on ice and add the following to the 10µl reaction.

*Eco*R I 10X buffer (pg. 215)	3µl
*Eco*R I	1u/pmole of linker used
H_2O	to final volume 30µl

2. Incubate at 37°C for 1.5 hours.
3. Add 3µl of 0.2M EDTA.
4. Add 1 volume of TE-saturated phenol/chloroform. Vortex for 1 minute and centrifuge at 12,000 x g for 3 minutes.
5. Remove the aqueous phase to a fresh tube. (To increase recovery, the phenol:chloroform phase can be re-extracted with a small volume of TE buffer.)

Note: For directional cDNA cloning, a digestion with *Xba* I or *Not* I must be performed. This is most conveniently done after the digestion with *Eco*R I.

D. Removal of Unligated Linkers

1. Spin column procedure: Apply the restriction digestion reaction (Step C.5) to the top of the gel bed of a spin column (see Note 3). Place the column in the collection tube and centrifuge in a swinging bucket at 800 x g for 5 minutes. The eluted cDNA can either be used directly or precipitated with ethanol by adding 0.5 volume 7.5M ammonium acetate and 2.0 volumes of ethanol. Mix and leave at –20°C for 30 minutes.
2. Centrifuge at 12,000 x g for 15 minutes. Carefully pour off the supernatant, wash the pellet with 1ml 70% ethanol, dry briefly in a vacuum desiccator, and resuspend in an appropriate volume of TE buffer for further reactions.

E. Efficiency of Linker Ligation

1. To test the efficiency of linker ligation to a substrate DNA, the following simple experiment can be performed. Mix together:

ligase 10X buffer (pg. 215)	1µl
BSA (1mg/ml)	1µl
blunt-ended DNA (i.e., restricted plasmid DNA):0.2pmole ends (see Step B.1, above)	2µl
phosphorylated *Eco*R I linkers (Dilution of stock linkers can be done in H_2O.)	$0.01A_{260}$u
T4 DNA ligase (Weiss units)	2.5u
H_2O	to final volume 10µl

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III. Ligation of *Eco*R I Linkers to cDNA

(continued)

2. Incubate at 15°C for 6-18 hours.
3. Add 1µl of sample buffer (below) to the ligation reaction.
4. Load the sample on a 0.8% agarose gel (with EtBr). Next to the ligated sample, load an aliquot of the blunt-ended substrate DNA.
5. Efficient ligation of the linkers to the substrate DNA will cause a shift in the migration rate due to the addition of linker concatemers. The ligated substrate DNA will run more slowly than the unligated sample.

Notes:

1. It is important that cDNA samples be size-fractionated prior to ligation with linkers. Small-sized DNAs, less than 200bp, must be eliminated to ensure successful linker addition and cloning. This step can be accomplished with gel filtration (column fractionation or spin columns) or agarose gel electrophoresis followed by selective elution of particular-sized cDNAs. Sephacryl® S-400* spin columns exclude double-stranded DNA fragments larger than 271 base pairs.
2. Preparation of spin columns: Add Sephacryl S-400 slurry with a Pasteur pipette to a vertical column and allow the buffer to drain completely. The final height of the gel bed should reach the neck of the column at the lower "ring" marking (this is about 1.2ml of a 1:1 slurry). (Columns can be pre-packed to this point, rehydrated with buffer, sealed top and bottom, and stored at 4°C as a slurry.) Before use, allow the buffer to drain, place the column tip into the provided wash tube and centrifuge in a swinging bucket for 5 minutes after the rotor reaches 800 x g. Pour off the eluate and respin to remove excess buffer. Discard the wash tube and collected fluid. The column is now ready for immediate use. Hold the pipet tip within a few millimeters of the gel bed and add sample dropwise, slowly, to the center of the dried gel bed, being careful not to let sample come in direct contact with the sides of the tube. The optimal sample volume is 20µl to 60µl. Do not exceed a 60µl sample volume.

*Sephacryl is a registered trademark of Pharmacia, Inc.

Composition of Solutions

***Eco*R I methylase 10X buffer:**

1M	Tris-HCl, pH 8.0
0.1M	EDTA

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

Sample buffer:

50%	glycerol
0.5%	SDS
0.1%	bromophenol blue
10mM	EDTA

Ligase 10X buffer:

300mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

***Eco*R I 10X buffer:**

900mM	Tris-HCl, pH 7.5
500mM	NaCl
100mM	$MgCl_2$

*Buffer tablets available from Promega.

IV. Ligation of *Eco*R I Adaptors to cDNA

The *Eco*R I adaptor ligation system is designed for the efficient addition of *Eco*R I sites to the termini of blunt-ended DNA molecules. This system allows cDNA to be cloned into unique *Eco*R I sites of vectors such as λgt10 or λgt11.

In addition, cDNA synthesized with an *Xba* I or *Not* I primer-adaptor can be directionally cloned into the *Eco*R I-*Xba* I arms of the LambdaGEM®-2 and LambdaGEM®-4 vectors, or into the *Eco*R I-*Not* I arms of the λgt11 *Sfi-Not* vector (see Figure 2, pg. 202).

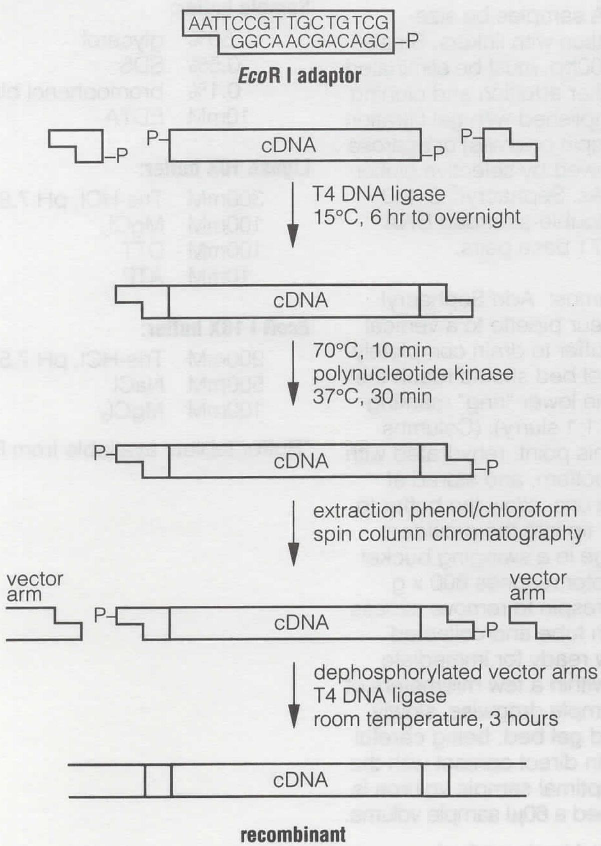

Figure 6. Schematic representation of *Eco*R I adaptor ligation system.

Adaptors eliminate the *Eco*R I methylation and restriction enzyme steps necessary with conventional linker addition methodology. The adaptor is a duplex DNA molecule with one blunt end (for ligation to cDNA) and an exposed *Eco*R I sticky end.

Ligation of adaptors to blunt-ended DNA (Figure 6) must be followed by a phosphorylation (kinasing) reaction if the cloning vector is dephosphorylated. The kinase reaction is unnecessary if the cloning vector is phosphorylated. In either case, excess adaptors can be quickly and efficiently removed with spin columns.

Reagents to be Supplied by the User

- TE buffer (pg. 217)
- TE-saturated phenol/chloroform (pg. 217)
- 7.5M ammonium acetate
- ethanol (100% and 70%)

A. Adaptor Ligation Procedure

1. To add *Eco*R I adaptors to a DNA fragment, set up the following reaction (the volumes can be scaled-up proportionately, depending upon the amount of DNA). If cDNA is used, see Note 1, pg. 217.

ligase 10X buffer (pg. 217)	3μl
bovine serum albumin (1mg/ml)	3μl
DNA (100ng/μl)*	2.5μl
*Eco*R I adaptors (20-fold molar excess: 10pmoles of adaptors)	1μl
T4 DNA ligase (Weiss units)	7.5u
H_2O	to final volume 30μl

*Example: 250ng of 1.5kb DNA (average size cDNA) = 0.5pmole ends

2. Incubate at 15°C for 6-18 hours.

3. Heat inactivate the reaction at 70°C for 10 minutes.

B. Kinase Reaction

A kinase reaction is required if the cloning vector is dephosphorylated, but is unnecessary if the vector is phosphorylated. Do not dissolve or precipitate DNA in the presence of ammonium ions prior to the kinase reaction.

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IV. Ligation of *Eco*R I Adaptors to cDNA

(continued)

1. Directly after the ligation reaction (pg. 216), cool the tube on ice and add the following to the 30µl reaction.

kinase 10X buffer (below)	4µl
0.1mM ATP (1:100 dilution in H_2O of 10mM stock:200pmoles)	2µl
T4 polynucleotide kinase (10 units)	1µl
H_2O	to final volume 40µl

2. Incubate at 37°C for 30 minutes.

   Note: For directional cDNA cloning, a digestion with *Xba* I or *Not* I must be performed. This can be done simultaneously with the kinase reaction. Substitute restriction enzyme 10X buffer for the kinase 10X buffer and increase the incubation time to 60 minutes.

3. Add 1 volume of TE-saturated phenol/chloroform (below). Vortex for 30 seconds and centrifuge at 12,000 x g for 3 minutes.

4. Remove the aqueous phase to a fresh tube. (To increase recovery, the phenol/chloroform phase can be re-extracted with a small volume of TE buffer.)

C. Removal of Unligated Adaptors

1. Spin column procedure: Apply the reaction mixture (Step B.4) to the top of the gel bed of a spin column (see Note 2). Place the column in collection tube and centrifuge in a swinging bucket at 800 x g for 5 minutes. The eluted cDNA can either be used directly or precipitated with ethanol by adding 0.5 volume of 7.5M ammonium acetate and 2.0 volumes of ethanol. Mix and leave at –20°C for 30 minutes.

2. Centrifuge at 12,000 x g for 15 minutes. Carefully pour off the supernatant, wash the pellet with 1ml of 70% ethanol, dry briefly in a vacuum desiccator, and resuspend in an appropriate volume of TE buffer for further reactions.

Notes:

1. It is important that cDNA samples be size-fractionated prior to ligation with adaptors. Small-sized DNAs (less than 200bp) must be eliminated to ensure successful linker addition and cloning. This step can be accomplished with gel filtration (column fractionation or even spin columns) or agarose gel electrophoresis followed by selective elution of particular-sized cDNAs. Sephacryl® S-400* spin columns exclude double-stranded DNA fragments larger than 271 base pairs.

2. Preparation of spin columns: Add Sephacryl S-400 slurry with a Pasteur pipette to a vertical column and allow the buffer to drain completely. The final height of the gel bed should reach the neck of the column at the lower "ring" marking (this is about 1.2ml of a 1:1 slurry). (Columns can be pre-packed to this point, re-hydrated with buffer, sealed top and bottom, and stored at 4°C as a slurry.) Before use, allow the buffer to drain, place the column tip into the provided wash tube and centrifuge in a swinging bucket for 5 minutes after the rotor reaches 800 x g. Pour off the eluate and respin to remove excess buffer. Discard the wash tube and collected fluid. The column is now ready for immediate use. Hold the pipet tip within a few millimeters of the gel bed and add sample dropwise, slowly, to the center of the dried gel bed, being careful not to let sample come in direct contact with the sides of the tube. The optimal sample volume is 20µl to 60µl. Do not exceed a 60µl sample volume.

Composition of Solutions

Ligase 10X buffer:

300mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

Kinase 10X buffer:

700mM	Tris-HCl, pH 7.6
100mM	$MgCl_2$
50mM	DTT

TE buffer*:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

TE-saturated phenol/chloroform:

Mix equal parts of TE buffer and phenol and allow the phases to separate. Then mix 1 part of the lower, phenol phase with 1 part of chloroform:isoamyl alcohol (24:1).

*Buffer tablets available from Promega.

cDNA Synthesis and Cloning

V. Ligation and Packaging *in vitro* of Insert DNA into Lambda Vectors

The construction of highly representative gene libraries is essential to successfully isolating cDNA or genomic clones from complex organisms. Bacteriophage lambda is an important cloning vehicle for this purpose primarily because of the high efficiency with which cells can be infected by phage particles packaged *in vitro*. Packaging *in vitro* refers to the use of a phage-infected *E. coli* cell extract to supply the mixture of proteins and precursors required for encapsidating lambda DNA.

The Packagene® extract system is derived from the unique one-strain host system described by Rosenberg (25,26). This system employs an *E. coli* C bacterial host to produce the extract. Since *E. coli* C lacks all known restriction systems, libraries constructed using the Packagene system are not biased by restriction of genetic material. In contrast, other commercially available packaging extracts are derived from *E. coli* K and initially contain both the K-12 and *mcr* restriction systems. While K-12 restriction can be inhibited, *mcr* restriction activity remains and can lead to loss of methylcytosine-containing DNA sequences from plant, mammalian, and other eukaryotic genomes.

The efficiency of packaging recombinant DNA using the single-strain Packagene extract is equivalent to that of two strain systems (*Promega Notes* 13). The extract contains all the components and buffers needed to perform the packaging reaction, and requires only the addition of substrate DNA. Control DNA (ligated concatameric λcI857 *Sam*7 DNA) is included for use in positive control packaging reactions. This allows evaluation of the Packagene system performance without having to use sample DNA. The λcI857 *Sam*7 control DNA must be used with a host having the *sup*F58 mutation. *E. coli* LE392 frozen in 20% glycerol is included as the specific host strain for the control DNA.

Summary

LB plates are prepared the day before ligation and packaging is to be performed. Allow 3.5 hours to complete the ligation of insert DNA with lambda vector DNA and an additional 3-4 hours for packaging and plating of the ligated DNA (15,25,26). Titrations of the cloning and packaging efficiencies can be determined the following day. Positive control DNAs are provided for testing of the cloning and packaging efficiencies (see Note 1, pg. 220). Multiple bacterial strains are provided with each lambda vector (see Table 3, pg. 204). Refer to pg. 405 in the Technical Appendix for help in selecting the best strain for each application.

Reagents Needed

Instructions for preparing these reagents are provided on pg. 221.

- ligase 10X buffer
- T4 DNA ligase
- phage buffer
- chloroform
- S buffer
- DMSO
- LB medium and plates
- supplemented LB medium
- TB top agar

A. Ligation of Positive Control Insert DNA

1. Prepare the host bacteria (see Note 2, pg. 220). Best results are obtained with a fresh bacterial culture which has been grown to an OD_{600} of between 0.6 and 0.7. Pick a single colony from a <u>freshly</u> streaked plate and inoculate into 3ml of supplemented LB medium (pg. 221). Shake at 37°C for approximately 5 hours and store at 4°C once the OD_{600} has reached 0.6.

   Alternatively, inoculate a single colony into 3ml (or up to 50ml) of supplemented LB media and shake overnight at 37°C. The next day, prepare a secondary culture by inoculating 500µl of the overnight culture into 50ml of supplemented LB media. Shake at 37°C for approximately 2.5 hours or until the OD_{600} has reached 0.6. Store at 4°C until use.

2. Prepare the ligation reaction. **Note:** To determine the optimal ligation conditions for a cDNA or genomic insert, see Step D, pg. 220.

lambda vector DNA (0.5µg)	1µl
restriction digested positive control insert DNA (0.2µg, see Note 1, pg. 220)	1µl
H_2O	2µl
ligase 10X buffer (pg. 221)	0.5µl
T4 DNA ligase (1.0 Weiss unit)	0.5µl
total volume	5µl

3. Incubate the ligation mixture for 3 hours at room temperature.

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V. Ligation and Packaging *in vitro* of Insert DNA into Lambda Vectors

(continued)

B. Packaging of Ligated DNA using the Packagene® *in vitro* Packaging System

1. Thaw the Packagene extract on ice. **Note:** The volume of the Packagene extract is 50µl.

2. As soon as the extract is thawed, add the entire ligation reaction (Step A.2) to the extract and mix by gently tapping the bottom of the tube several times. Do not add more than 10µl of ligation products per 50µl of packaging extract. (To determine the efficiency of the Packagene reaction alone, add 0.5µg of the provided concatemeric lambda *c*I857 *Sam*7 DNA directly to the extract and mix.)

3. Incubate at 22°C for 2 hours.

4. To the packaging mix (55µl), add phage buffer (pg. 221) to 0.5ml plus 25µl of chloroform (vector DNA concentration = 1µg/ml, if 0.5µg was packaged). Mix gently by inversion and allow the chloroform to settle to the bottom of the tube. The packaged phage can then be stored at 4°C for up to 3 weeks, although the titer may drop several fold under these conditions.

   For long term storage of packaged phage, add S buffer (pg. 221) instead of phage buffer and perform the chloroform extraction as described above. Add dimethylsulfoxide (7% final concentration) to the aqueous supernatant and store at –70°C.

C. Titration of Packaged Phage on LB Plates

1. Make appropriate dilutions of the packaging extracts (from Step B.4) in phage buffer. As a general guideline, an appropriate dilution for recombinant phage is 1:1,000 or 1:10,000.

2. Add 100µl of the diluted phage to 100µl of prepared bacterial cells (Step A.1, pg. 218) and allow the phage to adsorb for 30 minutes at 37°C. The λcI857 *Sam*7 control DNA must be used with a host that contains the *sup*F58 mutation. Both LE392 and KW251 contain this mutation.

3. Add 3ml molten (45°C) TB top agar (pg. 221). (To screen for recombinants by color selection, X-Gal and IPTG should be included in the top agar.) Vortex gently and immediately pour onto LB plates. Allow the top agar to harden and incubate inverted at 37°C overnight. (Best results are obtained by using fresh plates which have been allowed to dry overnight at room temperature so that they lose excess moisture.)

4. Count the number of plaques and calculate the titer of the phage. For example: if there are 20 plaques on a plate made from a 1/10,000 dilution, that means there are $20 \times 10{,}000 \times 10 = 2 \times 10^6$ plaque forming units (pfu) per milliliter of the original packaging extract. (The last 10 of the calculation converts the 0.1ml of the packaging extract which was plated to a per milliliter basis.) Since the vector DNA being packaged was at 1µg/ml, the efficiency of the arms is calculated to be 2×10^6 recombinants per microgram.

cDNA Synthesis and Cloning

V. Ligation and Packaging *in vitro* of Insert DNA into Lambda Vectors

(continued)

D. Determination of the Optimal Ligation Conditions for DNA Inserts with Lambda Vectors

1. To determine the optimal ligation conditions for a cDNA insert, proceed with the following small scale reactions shown in Table 5. Determining optimal ligation conditions is optional, but is encouraged if excess amounts of cDNA are available.

   We recommend that the molar concentration of the lambda vector remain constant while adjusting the amount of insert DNA. In the examples given, the molar ratio of lambda arms (43kb) to cDNA insert (average 1.5kb) varies between approximately 1:1-1:0.25. A tube lacking insert DNA is also used to determine background levels of religated arms.

2. Ligate and package DNA as indicated above (Steps A-C, pgs. 218-219).

3. Compare the titers from the various plates to determine the optimal relative concentrations of vector arms and DNA insert. The yield of recombinant plaques (from tubes B to D) should be approximately 100- to 1,000-fold greater than the yield of nonrecombinant plaques on the control plate (from tube A). Prepare a large scale reaction based on the optimum ratio of insert to vector DNA determined above. The amount of ligated DNA added to a Packagene extract in a large scale reaction may range between 0.5µg-5µg (suspended in a volume of 5-10µl).

Table 5. Optimization of Lambda: cDNA Insert Ligation.

	TUBE A	B	C	D
Vector DNA (0.5µg/µl; 1µg = 0.035pmole)	2µl	→	→	→
Insert DNA (10ng/µl; 10ng = 0.01pmole)	—	3µl	2µl	1µl
Ligase 10X Buffer	1µl	→	→	→
H_2O	6µl	3µl	4µl	5µl
T4 DNA Ligase (1-2 Weiss units)	1µl	→	→	→

Notes:

1. The positive control insert DNAs provided for tests of cloning and packaging efficiency are as follows:

Vector	Positive Control DNA
LambdaGEM-2* and LambdaGEM-4* vectors	pGEM-3* plasmid (2867 bp), digested with *Eco*R I and *Xba* I
λgt10 and λgt11 vectors	pSP64 plasmid (2999 bp), digested with *Eco*R I
λgt11 *Sfi-Not*	pGEM-9Zf(-)* plasmid (2925 bp), vectors digested with *Eco*R I and *Not* I.

(*U.S. Patent No. 4,766,072)

Concatemeric lambda *c*I857 *Sam*7 DNA is provided for tests of packaging efficiency alone.

2. The *E. coli* strain LE392 [F′, *hsd*R514 (r_k^-,m_k^-), *sup*E44, *sup*F58, *lac*Y1, *gal*T22, *met*B1, *trp*R55, λ-] is provided as a host strain to test the efficiency of the Packagene® system. This strain can also be used for screening and amplification of cDNA libraries, and is described more fully in the Technical Appendix (pg. 412).

3. The Packagene extracts can only tolerate one freeze/thaw cycle. A second freeze/thaw cycle will inactivate the product.

4. The volume of the Packagene extracts can be divided among several test reactions. For optimal activity, the entire 50µl should be used in constructing gene libraries.

5. The level of endogenous background plaques in a Packagene extract comprises less than 0.0001% of the expected phage yield.

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V. Ligation and Packaging *in vitro* of Insert DNA into Lambda Vectors

(continued)

Composition of Solutions

Ligase 10X buffer:

300mM	Tris-HCl, pH 7.8
100mM	$MgCl_2$
100mM	DTT
10mM	ATP

Phage buffer:

20mM	Tris-HCl, pH 7.4
100mM	NaCl
10mM	$MgSO_4$

LB (Luria-Bertaini) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust to pH 7.5 with NaOH and autoclave.

Supplemented LB medium:

50ml	LB medium
0.5ml	20% (v/v) maltose
0.5ml	1M $MgSO_4$

LB plates:

Add 15 grams of Bacto-agar to one liter of LB medium. Autoclave. Pour 30-35ml of medium into 85mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

TB top agar (100ml):

1.0g	Bacto-tryptone
0.5g	NaCl
0.6g	agar

Microwave or autoclave to melt the agar. When the solution has cooled to 60°C, add 1ml of 1M $MgSO_4$. For color selection of recombinants in conjunction with bacterial host strain Y1090, add 0.7ml IPTG (at 20mg/ml in H_2O, filter-sterilized) and 0.7ml X-Gal (at 50mg/ml in dimethylformamide) to the cooled (60°C) top agar.

S buffer:

Phage buffer + 2% (w/v) gelatin

VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

A. Description

Lambda expression vectors have become increasingly popular for the construction of cDNA libraries, specifically because of their ability to express high levels of desired products as proteins. Cloning into the *lacZ* gene can result in the expression of foreign DNA as part of a β-galactosidase fusion protein which can be detected with antibodies. The ProtoBlot® immunoscreening system allows the researcher's rabbit or mouse primary antibodies to be used as probes to detect recombinants containing DNA sequences of interest.

The ProtoBlot® immunoscreening system consists of the highest quality affinity purified second antibody alkaline phosphatase conjugates and the necessary color development substrates for the greatest sensitivity and maximum signal-to-noise ratios. A positive control is included consisting of a recombinant λgt11 ovalbumin clone and mouse monoclonal antibody. Also included is an *E. coli* extract for background reduction in cases where the primary antisera cross-reacts with *E. coli* antigens.

In a typical immunoscreening, host cells are infected with a recombinant λgt11 or λgt11 *Sfi-Not* library and plated as usual (17). After 3.5 hours of growth at 42°C, the plates are overlaid with nitrocellulose filters saturated with IPTG, an inducer of *lacZ* gene expression, and incubated for another 3.5 hours at 37°C. During this time, the plaques are transferred to the filters along with β-galactosidase fusion proteins released from lytically infected cells. The filters are removed from the plates and blocked by incubation in a solution containing excess protein to saturate nonspecific protein binding sites on the nitrocellulose. The filters are then incubated with a primary antibody directed against the antigen of interest, washed, and incubated with the appropriate (anti-rabbit or anti-mouse) second antibody alkaline phosphatase conjugate. After a final wash, color development substrates are added. Usually within a few minutes, positive plaques turn a dark purple color as a result of alkaline phosphatase activity. Positive plaques are then located and removed from the original plates and the recombinants plaque purified by repeating the same screening procedure until all plaques are positive.

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VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

(continued)

B. Protocol for Immunoscreening Lambda Expression Libraries

Reagents to be Supplied by the User

Instructions for preparing these reagents are supplied on pg. 227.

- LB medium, plates and top agar
- lambda diluent
- 10mM IPTG in H_2O
- TBST buffer
- blocking agent (e.g., calf serum, BSA, gelatin, casein)
- AP buffer
- AP color development substrate solution
- stop solution

(adapted from T. Huynh, R. Young and R. Davis, 17)

1. Streak out *E. coli* Y1090 for single colonies on LB plates, pH 7.5, containing 100µg/ml ampicillin and 15µg/ml tetracycline. Incubate at 37°C overnight. Starting with a single colony, grow Y1090 to saturation in LB medium (pg. 227) at 37°C with good aeration.

2. If plating is to be done on 100mm plates, mix 0.2ml of the Y1090 culture with 0.1ml of lambda diluent containing 3×10^4 pfu of the λgt11 or λgt11 *Sfi-Not* library for each plate. If plating is to be done on 150mm plates, use 0.6ml of the Y1090 culture with up to 10^5 pfu in lambda diluent for each plate. Adsorb the phage to the cells at room temperature for 20 minutes.

3. Add 2.5ml (for a 100mm plate) or 7.5ml (for a 150mm plate) of LB top agar, pH 7.5, to the culture and pour onto an LB plate, pH 7.5. Use slightly dry plates (i.e., 2 days old) so that the top agar will stick to the plate. Incubate the plates at 42°C for 3.5 hours.

4. Move the plates to a 37°C incubator. Carefully (avoiding air bubbles) overlay each plate with a dry nitrocellulose filter disk which has been saturated previously in 10mM IPTG in H_2O. Incubate for 3.5 hours at 37°C. (If a duplicate is required, a second filter may be overlaid after the first has been removed. Incubate for an additional 3 hours at 37°C.)

   Note: Incubation with the nitrocellulose filter may also be performed overnight at 4°C or, if the plaques are not yet large enough, at room temperature.

5. Move the plates to room temperature, quickly mark the position of the filter on the plate with a needle, and carefully remove the filters. If the top agar begins to stick to the filter rather than to the bottom agar, chill the plates at 4°C for 20 minutes. Place the filters in TBST buffer (pg. 227) and rinse briefly to remove any remnants of agar.

   Note: Do not allow the filters to dry out during any of the subsequent steps. At this point, the damp filters may be wrapped in plastic, then in foil, and stored at 4°C overnight. Perform all of the following washing and incubation steps at room temperature with gentle shaking. It is convenient to perform the incubations and washes in plastic petri dishes that are slightly larger than the filters. Use one filter per dish, with the surface that was in contact with the agar facing up.

6. To saturate nonspecific protein binding sites, incubate the filters in TBST + 20% calf serum or other suitable protein blocking agent (e.g., 1% BSA, gelatin, or casein) for 15 to 30 minutes. Use 7.5ml per 82mm filter and 15ml per 132mm filter.

7. Incubate the filters for 30 minutes in TBST + primary antibody. Use 7.5ml per 82mm filter or 15ml per 132mm filter. Use serum antibody or ascites fluid at a 1:200-1:1,000 dilution. The positive control mouse anti-β-galactosidase antibody can be used at a 1:5,000 dilution. The diluted primary antibody can generally be reused several times.

VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

(continued)

Notes:

a. The quality of the antibody probe is very important. High titer, high affinity antibodies produce better signals than low titer, low affinity antibodies. A purified IgG fraction will sometimes work better than whole antiserum and an affinity-purified antibody generally produces the best results. In general, antibodies that produce good signals on Western blots will produce good signals in the screening procedure at similar dilutions.

b. If little or no signal is obtained or if the background is too high in the screening procedure, the antiserum or ascites should be checked on Western or "dot" blots before proceeding. (The ProtoBlot immunoscreening system anti-rabbit and/or anti-mouse antibodies and substrates can be used as described here for these types of blotting.) A few antibodies, particularly some monoclonals, are conformation-dependent and do not recognize antigens immobilized on a surface. Others may tend to stick nonspecifically to surfaces such as nitrocellulose. These antibodies are not suitable for immunoscreening with this technique.

c. Most primary antibody preparations can be used in the 1:1,000 dilution range. Unnecessarily high concentrations of some antisera can accentuate backgrounds produced by anti-*E. coli* antibodies which may be present at low titers. (See Section C for the use of the *E. coli* extract for background reduction.)

8. Wash the filters 3 times, 5-10 minutes each, in 15-20ml TBST buffer.

9. Transfer the filters to TBST containing the appropriate second antibody-alkaline phosphatase conjugate. (A 1:7,500 dilution is recommended.) Allow 7.5ml per 82mm filter and 15ml per 132mm filter. Incubate for 30 minutes at room temperature.

10. Wash the filters in TBST as in Step 8.

11. Blot the filters damp dry on filter paper and transfer to freshly prepared AP color development substrate solution (pg. 227). Positive clones will appear as purple plaques on the white filters. Development of color will continue for at least 4 hours. Filters can be developed overnight, but may have high backgrounds as a result.

12. When the color has developed to the desired intensity, stop the reaction by replacing the substrate solution with stop solution (pg. 227). The filters can be stored in this solution or dried. The color will fade slightly upon drying but can be restored by moistening the filter with water.

13. To retest a putative positive signal, remove an agar plug containing phage particles from the region of the plate corresponding to the signal on the filter. Incubate the agar plug in 1ml of lambda diluent for at least 60 minutes at room temperature, vortexing occasionally. Replate the phage and repeat the screening procedure with the antibody probe until all the plaques on the plate produce a signal.

C. Background Reduction using *E. coli* Extract

Most rabbit antisera (and mouse ascites fluids) contain IgG components that bind to *E. coli* proteins. If the titer and/or binding affinity of these endogenous antibodies is high relative to the antibodies of interest, it may be difficult to distinguish true positives from background staining of other plaques.

Although our tests have shown that most antisera produce acceptable signal-to-noise ratios, some weakly binding low titer antisera may require treatment with the *E. coli* extract to reduce interference caused by cross-reacting components. In the majority of cases (where treatment is not absolutely necessary) the extract may be used to improve signal-to-noise ratios.

As noted in Section B, this type of background tends to increase with increasing concentrations of antiserum. Therefore, it is best to determine the least amount (highest dilution) of antiserum that still gives maximal signals with positive plaques. This will automatically give the minimum background possible for that antiserum without using the *E. coli* extract, and this will allow you to determine whether treatment with the extract is necessary. In general, the amount of extract needed to prevent background staining will depend on the dilution of antiserum. Higher concentrations of antiserum will require the use of more extract to absorb cross-reacting components.

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VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

(continued)

Treatment of Antisera with *E. coli* Extract

Incubate the diluted primary antibody in a solution containing an appropriate amount of *E. coli* extract for 30 minutes before adding the filter to be screened. As a guideline, we have found that a 1:1,000 dilution of antiserum requires about 1mg/ml of extract protein to effectively reduce the background. This same solution can be used for screening several filters and can be stored at –20°C, although multiple freeze/thaw cycles should be avoided.

Larger amounts of antiserum can be treated at once by immobilizing the extract on a solid support, such as agarose beads, and performing either a batchwise or column adsorption. To prepare an immobilized extract, first transfer it into 0.1M $NaHCO_3$, pH 8.5-9.0, 0.5M NaCl by dialysis or gel filtration. It can then be bound to CNBr-activated agarose or other activated matrices following the manufacturers' instructions.

D. Use of the Positive Control

The ProtoBlot immunoscreening system positive control consists of two separate components: a phage mixture containing a λgt11 recombinant and λgt10 in a 1:500 ratio and a mouse monoclonal anti-β-galactosidase antibody. The λgt11 recombinant contains ovalbumin cDNA which is expressed as a β-galactosidase/ovalbumin fusion protein. This fusion protein can be detected with the anti-β-galactosidase antibody. Excess λgt10 is provided in the phage mixture so that the ratio of positive to negative plaques more closely reflects that encountered in an actual screening experiment. Since λgt10 does not produce β-galactosidase, any signal obtained from these plaques represents the background to be expected when using high titer, high affinity antibodies having minimal levels of components which bind other *E. coli* proteins. This background signal is extremely low and often undetectable with the ProtoBlot immunoscreening system positive control.

Procedure for Making Positive Control Filters

1. Streak out *E. coli* Y1090 for single colonies on LB plates, pH 7.5, containing 100µg/ml ampicillin and 15µg/ml tetracycline. Incubate at 37°C overnight. Starting with a single colony, grow Y1090 to saturation in LB medium at 37°C with good aeration.
2. Add 0.3ml of lambda diluent to each of two sterile tubes. Make two serial 1:100 dilutions by adding 3µl of the positive control phage mixture to the first tube, mixing, and adding 3µl from this tube to the second tube.
3. If plating is to be done on 100mm plates, mix 0.15ml of the Y1090 culture (Step D.1) with 0.1ml of the diluted positive control phage (second tube). When using 150mm plates, mix 0.45ml of the Y1090 culture with 0.3ml of diluted positive control phage. Let the phage adsorb to the cells by incubating at room temperature for 20 minutes.

   The rest of the procedure is performed exactly as described in Steps B.3-B.12 (pgs. 222-223). A 1:5,000 dilution of the positive control mouse anti-β-galactosidase should be used as the primary antibody. The anti-mouse second antibody alkaline phosphatase conjugate should be diluted 1:7,500.

VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

(continued)

E. Troubleshooting

Symptoms	Possible Causes	Comments
Plaque problems	Plaques smear over plate.	The plates were too wet at plating. Remove moisture by leaving the tops off of the plates for 10-15 minutes. This can be done soon after plating at 42°C if excess water "pools" on the surface of the plates.
	Plaques are too small.	The plates may be too dry. Alternatively, the cell density may be too high; reducing the number of plating cells will increase the plaque size.
	Plaques are small on one side of the plate, large on the other side.	The top agar was not spread evenly.
Signal is weak or absent.	Antibody lost activity during storage or is of low titer.	Store antisera/ascites at –20°C in aliquots: avoid repeated freeze/thaw cycles. Use some other immunochemical assay, such as ELISA, immunoprecipitation, immunodiffusion, etc., to check reactivity toward the antigen. If positive, repeat the immunoscreening assay using higher concentrations of primary antibody.
	Antibody is of low affinity.	Low affinity antibodies are more affected by buffer conditions, incubation times, and relative concentrations than are high affinity antibodies. Eliminate Tween 20 from the buffers, increase the incubation time and concentration of the primary antibody.
	Antibody is conformation-dependent.	Refer to Section B.7 (pg. 223).
	Anti-IgG conjugate activity is too low.	Conjugate may have been improperly stored. Store at 4°C or in aliquots at –20°C. Avoid repeated freeze/thaw cycles, heat treatment, and bacterial contamination. Test activity by adding 1ml of diluted conjugate in TBST to 1ml of color development solution. Intense purple color should appear within 5 minutes. (This is also a test for activity of the color development substrates.) Although unlikely, it is possible that some water may contain inhibitors. Use reagents of highest quality available.
	Improper blocking agent was used.	Some blocking proteins may have effects on antigen recognition by some antibodies. If this is suspected, try a different blocking protein such as BSA, casein, gelatin or another type of serum to saturate excess binding sites. Include controls omitting the primary antibody, the anti-IgG conjugate and both antibodies from the procedure to check for possible effects on background.

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VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

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Symptoms	Possible Causes	Comments
General purple background throughout the membrane	Color development reaction was too long.	Stop the reaction when the color has reached the desired intensity.
	Poor quality nitrocellulose.	Use a different source of nitrocellulose.
	Poor quality alkaline phosphatase conjugates.	Be sure to use affinity-purified ProtoBlot second antibodies.
	Improper blocking.	The blocking step incubation time can be increased if necessary. Some alternative blocking agents such as non-fat milk may contain alkaline phosphatase activity or IgG that bind conjugates. Perform controls by omitting the primary antibody and the anti-IgG conjugate from the procedure.
	Anti-IgG conjugate concentration too high.	Reduce the concentration of conjugate.
	Primary antibody sticks nonspecifically even to blocked membranes.	Perform the procedure using just a blocked membrane without plaques. A few IgG and other immunoglobulins (particularly IgM) that may have determinants recognized by the second antibody conjugates are especially "sticky" and may be difficult to use for immunoscreening.
Background localized; unexpected plaques appear to be positive.	Primary antibody contains components that react with *E. coli* proteins.	Refer to Section C (pg. 223).
	Host strain, phage library, or culture medium is contaminated with organisms producing alkaline phosphatase.	This possibility can be tested by omitting the primary antibody and anti-IgG conjugate incubations from the procedure. If color development is significant, there is contamination. If it is not practical to start over with uncontaminated materials, try to inactivate the alkaline phosphatase by either heating the plaque lift at 80°C for 20 minutes or incubating it in 0.1M acetic acid for 20 minutes prior to the blocking step. If successful, test the effect of the treatment on primary antibody binding.
	Anti-IgG alkaline phosphatase cross-reacts with bacterial proteins.	Preadsorb the diluted second antibody conjugate with an *E. coli* extract as described in the section on antibody probes.

VI. Immunoscreening of Lambda Expression Libraries with the ProtoBlot® Immunoscreening System

(continued)

Composition of Solutions

Lambda diluent:

10mM Tris-HCl, pH 7.5
10mM $MgCl_2$

IPTG:

10mM isopropyl β-D-thiogalactopyranoside in H_2O

TBST buffer:

10mM Tris-HCl, pH 8.0
150mM NaCl
0.05% Tween* 20

*Tween is a registered trademark of ICI, Inc.

Blocking solution:

20% calf serum (or other appropriate protein) in TBST

AP buffer:

100mM Tris-HCl, pH 9.5
100mM NaCl
5mM $MgCl_2$

LB (Luria-Bertaini) medium (per liter):

10g Bacto-tryptone
5g Bacto-yeast extract
5g NaCl

Adjust pH to 7.5 with NaOH. Autoclave.

LB plates (plus ampicillin and tetracycline):

Add 15 grams of Bacto-agar to one liter of LB medium. Autoclave. Allow the media to cool to 55°C and then add 100µg/ml ampicillin and 15µg/ml tetracycline (filter-sterilized). Pour 30-35ml of medium into 100mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

LB top agar (100ml):

1.0g Bacto-tryptone
0.5g Bacto-yeast extract
0.5g NaCl
0.8g Bacto-agar

Microwave or autoclave to melt the agar and add 1ml of 1M $MgSO_4$.

AP color development substrate solution:

5ml AP buffer
33µl NBT(nitroblue tetrazolium) stock solution
16.5µl BCIP(5-bromo-4-chloro-3-indolyl phosphate) stock solution

Add NBT solution to AP buffer, mix. Then add BCIP solution and mix again. Protect the solution from strong light and use within 1 hour of preparation. If either of the substrates has precipitated during storage, warm the substrate vial to room temperature and mix to redissolve before use.

Stop solution:

20mM Tris-HCl, pH 8.0
5mM EDTA

VII. Preparation of Lambda Lysates and Isolation of Lambda DNA

The protocols listed below are provided in the Detection, Purification and Labeling chapter (pgs. 136-140).

A. Plate Method for Phage Lysate Preparation

B. Liquid Culture Method for Phage Lysate Preparation

C. Isolation of Lambda DNA with LambdaSorb® Phage Adsorbent

D. Miniprep Isolation of Recombinant Lambda DNA

cDNA Synthesis and Cloning

VIII. Preparation of Fusion Protein Extracts from λgt11 and λgt11 *Sfi-Not* Lysogens

A. Generation of Lambda Recombinant Lysogens in Y1089

It is often necessary to have preparative amounts of polypeptide specified by a cloned piece of DNA in lambda expression vectors such as λgt11and λgt11 *Sfi-Not*. A crude lysate containing a particular recombinant protein can be prepared easily by expressing the desired lambda lysogen in *E. coli* Y1089 (17). Accumulation of large amounts of a fusion protein in this strain is possible because of a mutation which enhances the frequency of phage lysogeny (*hfl*A150). The general strategy is as follows: first, screen the recombinant cDNA library in the bacterial strain Y1090. Once the desired clone is detected, isolate the recombinant phage and infect Y1089 with the clone of interest. The lysogen is grown to high cell density, *lac*Z-directed fusion protein production is induced by the addition of IPTG to the medium and the cells are harvested and lysed.

Reagents to be Supplied by the User

- LB medium and plates (pg. 229)
- phage buffer (pg. 229)
- IPTG
- TEP buffer (pg. 229)
- saturated $(NH_4)_2SO_4$ solution

1. Grow Y1089 cells overnight at 37°C in LB medium (pg. 229), pH 7.5, supplemented with 0.2% (v/v) maltose and 10mM $MgCl_2$. Read the OD_{600} of the culture and dilute the cells to 1 x 10^8 cells/ml. (An OD_{600} of 1.0 is equivalent to 8 x 10^8 cells/ml.)
2. Infect 10-100µl of cells with the recombinant phage at a multiplicity of infection of approximately 5. Incubate for 20 minutes at 32°C. Recombinant phage may be obtained directly from an isolated phage plaque that has been allowed to diffuse out of the agar into 1.0ml of phage buffer (pg. 229). An average phage plaque yields 10^6-10^7 infectious phage particles.
3. Dilute the infected cells in LB medium supplemented with 10mM $MgSO_4$ and plate them on LB plates at a density of approximately 200 per plate. Incubate at 32°C. At this temperature, the temperature-sensitive phage repressor is functional.
4. Test 20 single colonies by the following method. Using a sterile toothpick, pick a single colony and streak onto two LB plates. Incubate one plate at 32°C and the other at 42°C.
5. Clones which show confluent growth at 32°C but spotty growth at 42°C are assumed to be lysogens (27). Lysogens arise at a frequency between 10-70%.

B. Preparation of a Crude Lysate from a Lambda Lysogen

1. Inoculate 100ml of LB medium, pH 7.5, with a single colony of the Y1089 recombinant lysogen. Incubate the culture at 32°C with good aeration.
2. When the culture has grown to an OD_{600} = 0.5, increase the temperature of the culture to 42°- 45°C as rapidly as possible, and incubate the culture at the elevated temperature for 20 minutes with good aeration.
3. Add IPTG to 10mM.
4. Incubate the culture at 37°- 38°C for approximately 60 minutes. (Do not let the temperature of the culture drop below 37°C.)

   Note: At this stage, the Y1089 lysogen will sometimes lyse, even though Y1089 does not suppress the mutation causing defective lysis (*S*100) in λgt11 and λgt11 *Sfi-Not*. This is a consequence of 2 factors: 1) the *S*100 amber mutation is "leaky", and 2) the accumulation of foreign proteins in *E. coli* often renders it susceptible to lysis. For this reason, the longest incubation time achievable at 37°- 38°C without lysis occurring should be determined for each individual recombinant lysogen.
5. Harvest the cells as rapidly as possible by centrifugation at 3,000 x g for 5 minutes at 24°- 37°C. (A sudden shift in temperature during harvest appears to increase the rate of proteolysis in experiments done on a small number of different lysogens. Therefore, centrifugation is performed between 24°C and 37°C.)
6. Rapidly resuspend the cells in 1/20 to 1/50 of the original culture volume in a buffer suitable for proteins. We suggest resuspending the cells in TEP buffer (pg. 229).

Promega

VIII. Preparation of Fusion Protein Extracts from λgt11 and λgt11 *Sfi-Not* Lysogens

(continued)

7. Immediately freeze the resuspended cells in liquid nitrogen or on dry ice and store at –70°C.

8. Thawing of the frozen cells results in lysis of the induced lysogen. However, sonication is also recommended to completely lyse the extract and to reduce the viscosity of the extract.

9. Centrifuge the sonicated extract at 11,000 x g for 10 minutes. Decant the supernatant to a separate tube.

10. Add 3 volumes of a saturated $(NH_4)_2SO_4$ solution to each volume of supernatant. Mix well and chill at 4°C for 60 minutes. The extract should be stored as a slurry at 4°C.

11. Follow the protocol described on pgs. 229-231 for the purification of fusion proteins using ProtoSorb *lac*Z immunoaffinity adsorbent.

Composition of Solutions

LB (Luria-Bertani) medium (per liter):

10g	Bacto-tryptone
5g	Bacto-yeast extract
5g	NaCl

Adjust to pH 7.5 with NaOH and autoclave.

LB plates:

Add 15 grams of Bacto-agar to one liter of LB medium. Autoclave. Pour 30-35ml of medium into 100mm petri dishes. If necessary, flame the surface of the medium with a Bunsen burner to eliminate bubbles. Let the agar harden. Store at room temperature (for 1 week) or at 4°C (for 1 month).

TEP buffer:

100mM	Tris-HCl, pH 7.4
10mM	EDTA
1mM	PMSF (phenylmethylsulfonyl fluoride diluted from a 100mM stock in ethanol)

Phage buffer:

20mM	Tris-HCl, pH 7.4
100mM	NaCl
10mM	$MgSO_4$

IX. Immunoaffinity Isolation of β-Galactosidase Fusion Proteins using ProtoSorb® *lac*Z Adsorbant

A. General Considerations

ProtoSorb® *lac*Z is an immunoaffinity adsorbent designed for the purification of β-galactosidase fusion proteins. The column matrix consists of a pure, specific mouse monoclonal anti-β-galactosidase antibody coupled to cross-linked agarose beads. This antibody has unique binding and release properties. Its affinity for β-galactosidase is high enough to allow for quantitative and specific binding of either N-terminal or C-terminal fusion proteins from crude cell extracts, yet low enough to allow efficient elution under relatively mild conditions. Bound proteins are eluted by raising the pH to 10.8 or, as an alternative, at pH 2.9 in conjunction with 3M urea. In a typical application using a 1ml column, 500µg of fusion protein can be purified from crude material in less than two hours with yields greater than 80%. Using the recommended elution conditions, the column can be recycled at least five times without significantly affecting performance. The adsorbent is available as a prepoured column with a 1ml bed volume or as a 5ml bulk size.

Procedures that yield the most efficient recoveries of fusion protein may differ depending on the expression system, the fused polypeptide, and the relative abundance of the fusion protein. Although the epitopes recognized by the anti-β-galactosidase monoclonal antibody have not yet been mapped, the adsorbent will bind both N- and C-terminal fusion proteins. In some systems, the expression of the fusion protein is induced with IPTG prior to cell lysis. Procedures for the generation and induction of lambda recombinant lysogens in strain Y1089 are described on pg. 228.

The characteristics of the polypeptide moiety fused to β-galactosidase may influence the method of preparation of cell extracts. For example, hydrophobic regions may cause a significant proportion of the fusion protein to be membrane associated in which case it would be desirable to include a nonionic detergent in all buffers. Antibody binding by the adsorbent is not affected by the presence of up to 1% Triton X-100, 1M NaCl or NP-40. Note, however, that NP-40 will interfere with absorbance readings at 280nm.

IX. Immunoaffinity Isolation of β-Galactosidase Fusion Proteins using ProtoSorb® *lac*Z Adsorbant

(continued)

The efficiency of recovery from any adsorbent is proportional to the number of sites occupied by the ligand. This is an important consideration, particularly when purifying small amounts (less than 50µg) of fusion protein. In general, the amount of adsorbent used should be adjusted so that more than 20% of the available binding sites will be occupied by a given amount of fusion protein. The exact binding capacity of the adsorbent under standard conditions can be found on the Certificate of Analysis provided with each order. In certain cases where the fusion protein is of extremely low abundance (i.e., less than 0.2% of the total protein), recovery may be improved by including a fractionation step prior to immunopurification. Examples of such procedures include gel filtration chromatography and back extraction of the original 75% ammonium sulfate precipitate (see below) with lower ammonium sulfate concentrations.

Reagents to be Supplied by the User

Instructions for preparing these reagents are supplied on pg. 231.

- TEP buffer
- Tris buffer
- TN buffer
- High pH elution buffer
- Low pH elution buffer
- TBS buffer

B. Sample Preparation

Detailed procedures for preparing a crude lysate from lambda recombinant lysogens are provided on pgs. 136-137. The final steps are summarized below.

1. Perform all steps at 4°C or on ice. Prepare a crude lambda sonicate in TEP buffer (pg. 231) and centrifuge at 10,000 x g for 15 minutes to remove unbroken cells and debris.

2. To the supernatant, add either 3 volumes of saturated ammonium sulfate or solid ammonium sulfate to 75% saturation and store at 4°C.

C. Binding and Elution of β-Galactosidase Fusion Proteins

The following procedure is designed for the use of the 1ml prepoured immunoaffinity column. The protocol can be modified for using smaller column volumes or homemade columns with the bulk adsorbent. For certain applications, especially when using small amounts of adsorbent, it may be desirable to add the beads to the extract and perform the procedure batchwise, using the same buffers as for column purification.

1. If the extract has been stored as an ammonium sulfate precipitate, centrifuge it at 10,000 x g for 20 minutes, remove the supernatant and redissolve the pellet in cold TEP buffer at approximately 20mg/ml protein.

2. Dilute the sample with Tris buffer (pg. 231) to approximately 4mg/ml total protein before loading the column.

3. Make sure that the column bed and dead space below the bed are free of bubbles which may have accumulated during shipping. To remove bubbles from the bed, seal the top and resuspend the column matrix by gentle mixing. If there is air trapped in the dead space, it can be removed using a syringe filled with Tris buffer.

4. Equilibrate the column with 5ml of cold Tris buffer. It is convenient to carry out the procedure in a container of ice, placing the column in 15ml disposable centrifuge tubes.

5. Adjust the flow rate so that 5ml of buffer will pass through the column in 30-60 minutes. This can be done by fitting the bottom of the column with a syringe needle (usually 25-26 gauge). The Luer fittings of some needles are too long to make a good seal. If this is the case, cut off the tip of the column with a razor blade.

6. Load the diluted extract and let it flow through the column. If the fused protein is fairly concentrated (100-300µg/ml), slower flow rates (i.e., 5ml/hour) or multiple passes are recommended.

IX. Immunoaffinity Isolation of β-Galactosidase Fusion Proteins using ProtoSorb® *lac*Z Adsorbant

(continued)

7. After loading, wash the column with 15ml of TN buffer. If a needle was used to restrict the flow rate during loading, it can be removed during the wash. Collect fractions and determine when the A_{260} reaches baseline.

8. The fusion protein may be eluted under several conditions, depending on the application. For the 1ml column, 3 successive 1ml aliquots of the elution buffer followed by 1ml of TBS (no NP-40) should be used, resulting in a total volume of 4ml of eluted sample. The most gentle elution condition for most proteins is 0.1M $NaHCO_3/Na_2CO_3$, pH 10.8, which should elute more than 80% of the bound protein. While the high pH elution is recommended, a low pH alternative eluant is 0.1M citrate/NaOH, 3M urea, pH 2.9, which should elute more than 60% of the protein. Note that β-galactosidase activity is destroyed by both of these elution buffers. Both of these conditions can be used for at least 5 cycles without significantly affecting column performance, provided that the column is quickly reequilibrated in a neutral buffer such as TBS. For storage, it is also recommended that 0.02% sodium azide be included in the buffer.

   The bound fusion protein can also be eluted under harsher conditions (e.g., 6M ammonium thiocyanate, 6M urea, or 6M guanidine HCl), but these conditions may be unnecessarily harmful to the column, making recycling impossible.

Composition of Solutions

TEP buffer (make fresh each time):

100mM	Tris-HCl, pH 7.4
10mM	EDTA
1mM	phenylmethylsulfonyl fluoride (PMSF, dissolved initially as a 100mM stock in EtOH)

Tris buffer:

50mM	Tris-HCl, pH 7.3

TN buffer:

50mM	Tris-HCl, pH 7.3
0.2%	NP-40

High pH elution buffer (0.1M $NaHCO_3/Na_2CO_3$, pH 10.8):

0.1M	$NaHCO_3$ = 0.42g in 50ml dH_2O
0.1M	Na_2CO_3 = 0.53g in 50ml dH_2O

Mix 50ml of each solution and adjust pH to 10.8 with NaOH.

Low pH elution buffer (0.1M Citrate/NaOH, 3M Urea, pH 2.9):

5ml	1M citric acid stock, pH 2.9
15ml	10M urea

Mix. Adjust the pH to 2.9. Bring volume up to 50ml.

TBS buffer:

50mM	Tris-HCl, pH 7.3
150mM	NaCl

cDNA Synthesis and Cloning

X. Reverse Transcription System for First Strand cDNA Synthesis

Promega's reverse transcription system provides a convenient means to efficiently reverse transcribe total or poly(A)+ RNA into cDNA for use in subsequent applictions (28). A polyadenylated 1.2kb transcript is provided as a control template for the cDNA synthesis reaction (see Note 1).

Reagents to be Supplied by the User

- sample buffer (pg. 233)

A. Standard Reverse Transcription Reaction

1. Prepare a 20µl reaction (for 1µg of RNA) by adding the following reagents in order: (Note that this reaction can be scaled up or down depending on the amount of RNA).

Component	Volume	Final Concentration in 20µl
25mM $MgCl_2$	4µl	5mM
reverse transcription 10X buffer (pg. 233)	2µl	1X
10mM dNTP mixture	2µl	1mM each dNTP
rRNasin® ribonuclease inhibitor	0.5µl	1u/µl
AMV reverse transcriptase (HC)	15u	15u/µg
oligo(dT)$_{15}$ primer	0.5µg	0.5µg/µg RNA
positive control RNA or substrate RNA	1µg	50ng/µl
RNase-free water	to final volume 20µl	

2. Incubate the reaction at 42°C for 15 minutes.

3. To analyze the product on a gel, proceed to Section C, below. **DO NOT** heat the reaction. Heating will cause the RNA/cDNA hybrid to denature, thus creating ambiguous gel results. For other applications, the sample should be heated at 99°C for 5 minutes followed by a 5 minute incubation at 0-5°C. This will inactivate the reverse transcription and prevent it from binding to the cDNA.

B. Dilution of the Reaction

1. The first strand cDNA reaction may be diluted in the following manner:

Component	Volume	Final Concentration in 100µl
first stand cDNA reaction	20µl	<10ng/µl
cDNA reaction dNTPs		200µM
25mM $MgCl_2$	4µl	1.5mM
reverse transcription 10X buffer	8µl	1X
RNase-free water	65.5µl	
diluted sample volume	97.5µl	

Promega

X. Reverse Transcription System for First Strand cDNA Synthesis

(continued)

C. Gel Analysis of First Strand cDNA Products

1. To examine the conversion of control RNA to cDNA, add 15µl of the reaction to 5µl of the sample buffer and load on a 1% agarose gel containing 0.5µg/ml ethidium bromide. Compared to a sample (approximately 250ng) of the control RNA template, there should be a band shift from the lower molecular weight RNA to the higher molecular weight RNA/cDNA hybrid.

Alternatively, a denaturing alkaline agarose gel may be used for gel analysis (15).

Notes:

1. The positive control RNA contains the sequence encoding kanamycin resistance. Heating the control RNA at 65°C for 5 minutes prior to loading on an agarose gel eliminates any secondary structure. If the sample is not heated, the control RNA tends to give a banding pattern of two bands rather than one uniform band.

2. The following reagents may be prepared as a stock just prior to setting up the reactions and aliquoted into individual tubes when needed: water, buffer, dNTPs, $MgCl_2$, rRNasin® ribonuclease inhibitor, and reverse transcriptase. This allows fewer pipetting steps and improves accuracy.

3. The suggested magnesium concentration may be optimized for any given sequence to achieve better yields.

4. Random primers at a concentration of 200ng primer/µg RNA may be substituted for the oligo(dT) primer in the reverse transcription reaction. When using random primers, incubate the reaction at room temperature for 10 minutes and then incubate at 42°C for 15 minutes. This additional incubation allows extension of the primers so that they remain hybridized when the temperature is raised to 42°C.

5. Specific downstream primers (provided by the user) may also be substituted for the oligo(dT) primer. The concentration of a specific primer should be adjusted according to the type of reverse transcription being performed. For example, when a 24mer primer is hybridized to 1.0µg of control template RNA, 800ng (100pmol) is required. When the identical primer is hybridized to a specific RNA in a total RNA sample, as little as 120ng (15pmol) is required. Specific primers are typically 19-30 bases long.

6. To obtain longer and/or more abundant transcripts the cDNA reaction may be incubated for up to 60 minutes at 42°C.

7. In cDNA synthesis, significantly fewer units of AMV reverse transcriptase are needed for cDNA synthesis relative to some other reverse transcriptases such as MMLV reverse transcriptase.

Composition of Solutions

Reverse transcription 10X buffer:

100mM	Tris-HCl (1X is pH 8.8 at 25°C)
500mM	KCl
1%	Triton X-100

Sample buffer:

2%	Ficoll
0.5%	SDS
50mM	EDTA
0.2%	orange G
	or
50%	glycerol
0.5%	SDS
0.1%	bromophenol blue
100mM	EDTA

XI. Construction of Subtraction Libraries: Overview and Schematic Diagram

(by M.J. Palazzolo and E.M. Meyerowitz, California Institute of Technology)

The isolation of tissue-specific genes in systems such as *Drosophila*, where subsequent genetic analysis is possible, would allow definitive studies aimed at understanding the physiology of differentiated cells. Subtraction hybridization is potentially the most sensitive technique for identifying mRNA molecules expressed in one tissue and not another. These procedures have been based on screening plaque lift filter replicas of cDNA and genomic libraries with labeled cDNA probes. Unfortunately, control experiments have shown that it is difficult to identify clones that are homologous to RNA molecules present in the probe at less that 1 part in 200 (15). Since most tissue-specific mRNAs are represented at much lower abundance, it is unlikely that these procedures will allow the isolation of their corresponding cDNAs. One reason for this logistical limitation of subtractive hybridization procedures has been caused by the requirement for large amounts of poly(A)+ RNA for probe synthesis. Since most tissue-specific sequences are extremely rare, large quantities of mRNA are needed in order to incorporate detectable amounts of label into these rare sequences among the total cDNA probe. Since most dissectable tissues in the fruit fly are quite small, this requirement has effectively limited the use of standard subtractive procedures.

The LambdaGEM®-2 and LambdaGEM-4 vectors (originally λSWAJ) were developed to allow the amplification of mRNA sequences. By orientation-specific cloning of cDNA between the T7 and SP6 promoters, it is possible to synthesize large amounts of sense or anti-sense "cRNA" molecules *in vitro* using DNA prepared from whole libraries.

The first advantage of this system is that a minimal amount of dissected tissue is required for the use of subtractive hybridization procedures. For example, a representative cDNA library (more than 1 million individual clones) can be constructed from one thousand imaginal discs. cRNA from this library can then be used in an essentially unlimited number of subtractive hybridizations. In contrast, using standard procedures, more than 200,000 discs would be necessary for a single subtractive hybridization.

Another advantage of the system is that both ends of the cRNA molecules contain defined nucleotide sequences so the cDNA copies can be easily generated using appropriate primers. Thus, it is possible to simply construct a cDNA library from the unhybridized molecules remaining after subtractive hybridization.

Furthermore, it is simple to synthesize high specific activity RNA probes directly from a subtracted library in which essentially all of the radioactivity is introduced into the rare cloned sequences. These probes can then be used in extremely sensitive assays (Northern blots, RNase protection) to identify rare tissue-specific sequences.

In situ hybridization to tissue sections requires high specific activity anti-sense probes made from individual clones, which are easily prepared from LambdaGEM recombinants without the need to determine orientation or to subclone. These probes can be used to directly screen for temporally- and positionally-specific transcription of specific genes.

The flanking T7 and SP6 promoters also serve as excellent priming sites for dideoxy sequence analysis. The ends of cDNA clones can be sequenced directly (without subcloning) using either RNA transcripts or double-stranded phage DNA as the template, with T7 and SP6 promoter oligonucleotide primers and the GemSeq® Transcript or *Taq*Track® Sequencing Systems, respectively.

XI. Construction of Subtraction Libraries: Overview and Schematic Diagram

(continued)

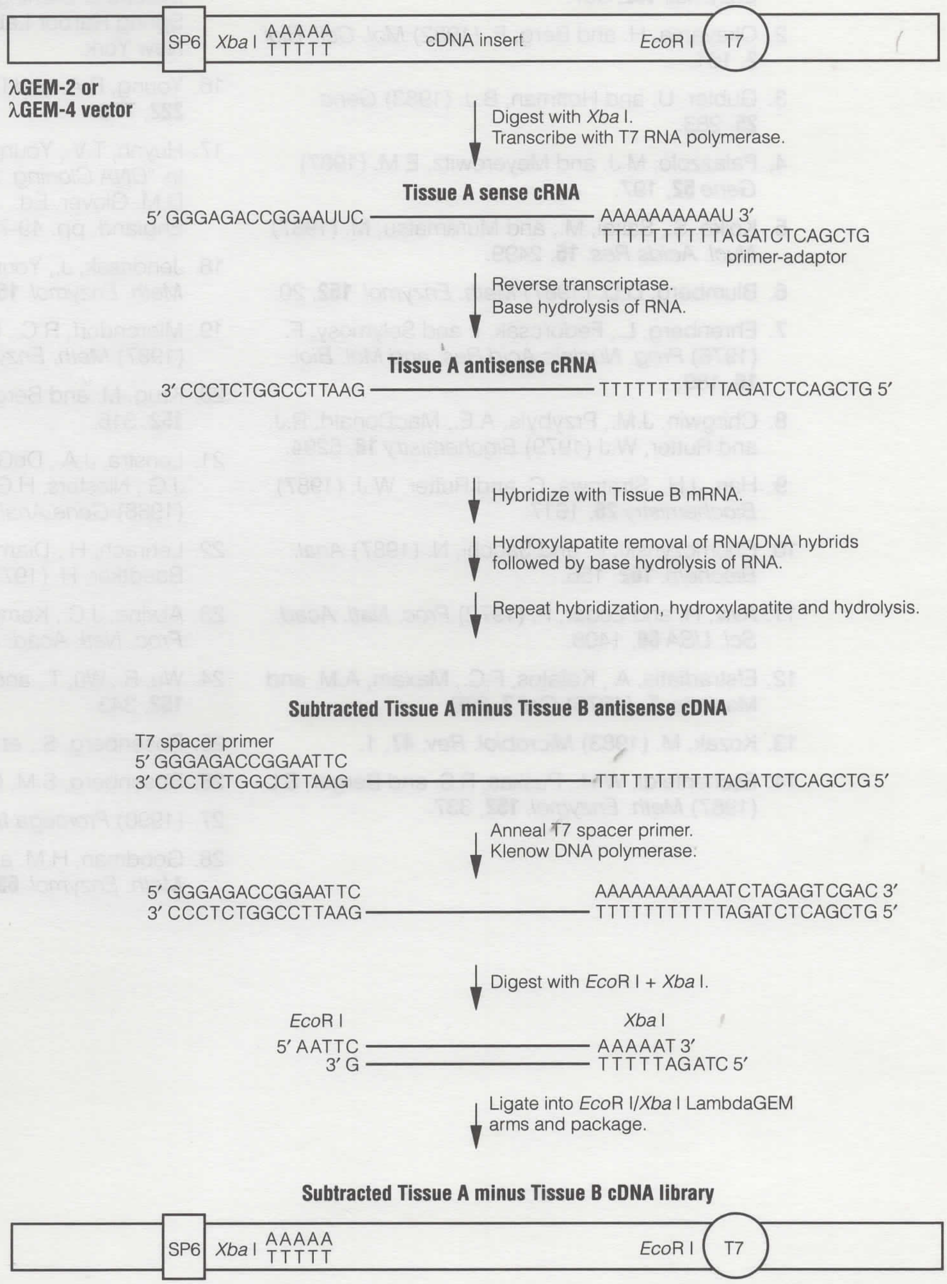

Figure 7. Schematic diagram of subtraction library construction.

cDNA Synthesis and Cloning

XII. References

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XIII. Additional cDNA Synthesis and Cloning Literature Available from Promega

Technical Bulletins

005	Packagene® *in vitro* Packaging System; User Information
006	Protoclone® λgt11 System; Positive Control Protocol
020	AMV Reverse Transcriptase; cDNA Synthesis
025	Protoclone® λgt10 System; Positive Control Protocol
027	Preparation of Fusion Protein Extracts from λgt11 Lysogens
051	LambdaGEM®-2, LambdaGEM®-4 *Eco*R I-*Xba* I Arms
054	Synthesis of Subtraction Hybridization RNA Probes Using LambdaGEM®-2 or LambdaGEM®-4 Vectors
056	Preparation of LambdaGEM®-2 or LambdaGEM®-4 Vectors for Cloning
062	*Eco*R I Linker Ligation System
066	LambdaGEM®-2 *Eco*R I Arms Protocol
067	RiboClone® *Eco*R I Adaptor Ligation System
072	Lambda gt11 *Sfi-Not* Vector (*Eco*R I-*Not* I Arms)
099	Reverse Transcription System

Manuals

ProtoBlot® Immunoscreening System Technical Manual

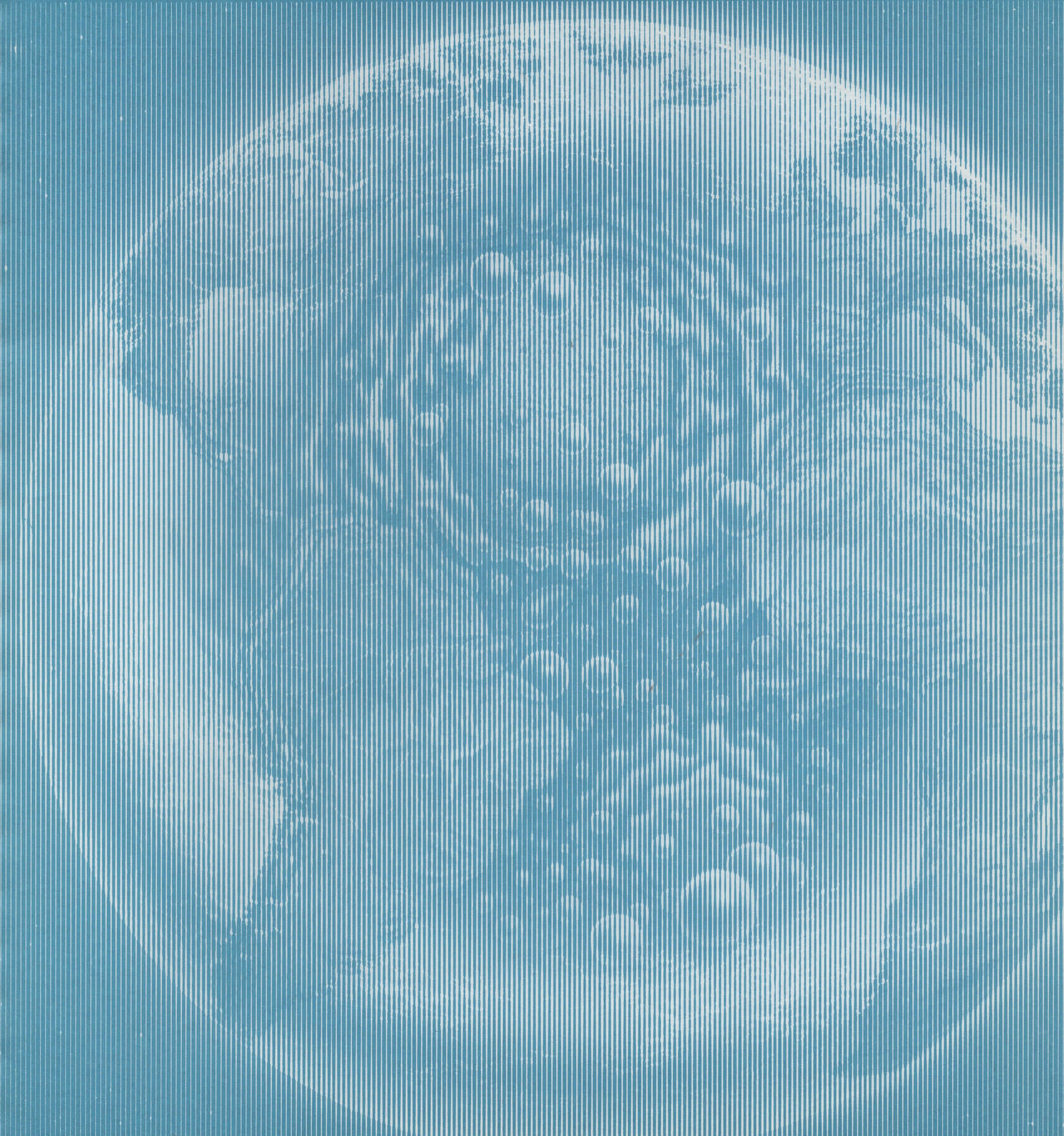
RiboClone® cDNA Synthesis Systems Technical Manual

Promega Notes Articles

Issue	Title
3	Cloning of the gene for the regulatory subunit of cAMP-dependent protein kinase in *Dictyostelium*
3	ProtoBlot® immunoscreening system
3	Monoclonal anti-β-galactosidase applications
4	Screening for lambda gt11 recombinant lysogens
4	Protoclone® lambda gt10 system: product update
5	ProtoSorb® *lac*Z immunoaffinity adsorbent
6	Rapid isolation of lambda DNA with LambdaSorb® phage adsorbent
9	Generation of random primed cDNA libraries
12	RiboClone® cDNA synthesis systems
13	An evaluation of *in vitro* packaging of lambda recombinants
14	cDNA cloning simplified: the RiboClone® *Eco*R I adaptor ligation system
17	cDNA update: increased first strand conversion for cDNA synthesis
17	Streamlined protocol for directional cloning using *Eco*R I adaptors
17	cDNA analysis on neutral gels
18	Gel electrophoresis of lambda DNA: NaCl phenomenon
22	Rapid, high yield isolation of total RNA using the RNAgents™ total RNA isolation kit
25	The PolyATtract™ magnetic mRNA isolation system: optimization and performance
25	Efficiency of packaging lambda DNA *in vitro* in the presence of tRNA
26	LambdaSorb® phage adsorbent update
26	Design of oligonucleotide probes: preparation of proteins for sequencing with the Probe-Design™ peptide separation system
27	Generation of recombinant lysogens in Y1089
27	Quantitation of β-galactosidase fusion proteins by a two-site ELISA procedure

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Protein Analysis

Contents

Protein Analysis

I. General Considerations for Protein Analysis

A. Gel Methods for Protein Analysis

When analyzing a protein sample, it is almost always necessary to obtain a small amount of the protein in purified form. In the past, purification was primarily accomplished by column chromatography. However, as techniques for protein detection and analysis have become more sensitive, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has become the method of choice for the isolation of small amounts of protein.

In this technique, the sample to be fractionated is denatured and coated with detergent by heating in the presence of SDS and a reducing agent. The SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on a polyacrylamide gel and high voltage is applied, causing the protein components to migrate towards the (+) electrode (anode).

Since all of the proteins have a net negative charge that is in proportion to their size, the proteins are separated solely on the basis of their molecular mass - a result of the sieving effect of the gel matrix. The molecular mass of a protein can be estimated by comparing the gel mobilities of a band with protein standards. Sharp banding of the protein components is achieved by using a discontinuous gel system, having stacking and separating gel layers that differ in either salt concentration or pH or both (1).

We routinely perform SDS-PAGE separations in a minigel format (about 7 x 9cm). We prefer minigel systems over larger gel formats because they have a sample capacity high enough to meet our needs (up to 4µg per band per lane), require considerably less acrylamide and buffer than larger systems, and allow more rapid run times. We routinely cast gels 0.75-1.0mM thick for general protein fractionation and recovery, and the protein analysis protocols in this chapter have been scaled for use with this type of gel.

Following gel electrophoresis, individual gel bands may be visualized and recovered from the gel by passive diffusion or electroelution. Visualization of gel-resolved proteins may be accomplished by a variety of methods, including incubation with KCl or sodium acetate solutions (2,3). However, the two most commonly used methods are Coomassie Blue and silver staining (4), which allow detection of protein bands in the range of 50ng and 5ng, respectively. Coomassie Blue is more often used due to its simplicity of use and because of published protocols using the dye that allow recovery of the protein bands for further analysis.

Both Coomassie Blue and silver staining methods require several hours to complete. In addition, proteins visualized by these methods are exposed to stringent conditions which can make them unusable or difficult to manipulate for protein sequence analysis, protein fingerprinting or for elution from the gel. The acidic conditions employed in Coomassie staining can cause a partial cleavage at Asp-Pro linkages (5). In addition, the acid/methanol staining solution strips SDS off of proteins. Recoating with SDS is thus necessary for efficient recovery of Coomassie-stained proteins from the gel. Silver staining is not appropriate when proteins are to be recovered from the gel because the conditions employed oxidize the protein.

Promega's ChromaPhor™ protein visualization system (pg. 245) overcomes these problems by staining proteins as the gel is running. Because the interaction between the dye and protein bands does not fix the protein into the gel or alter it chemically, the protein can be recovered readily and used in further analyses. Section II (pg. 245) contains a discussion of the several applications of the ChromaPhor system and a protocol for recovery of proteins from gel slices.

In some cases it is possible to isolate small amounts of active protein by nondenaturing, or "native", gel electrophoresis. Native gel electrophoresis is most commonly performed by eliminating the heating step in sample preparation and, sometimes, by eliminating SDS from the gel buffers. If denaturation is successfully avoided, the active protein may be detected in the gel by measurement of its enzymatic activity or other properties. When a protein's activity can not be preserved during electrophoresis, it is sometimes possible to recover activity by refolding in the presence of guanidine-HCl (2).

Gel-purified proteins may be used for a variety of applications, including antibody production and screening, protein sequence determination, and structural studies such as protein fingerprinting and epitope/modification mapping. Promega has developed systems and reagents which simplify many of these procedures, and protocols for the use of these systems are provided below.

Promega

I. General Considerations for Protein Analysis

(continued)

B. Peptide Generation and Separation

Some of the most powerful methods for the analysis of proteins are protein sequencing, protein fingerprinting, and mapping of epitopes and post-translational modifications. These approaches rely on a common strategy, that of partial or total cleavage of the target protein and separation of the resultant peptide fragments. Typically, SDS-PAGE has been used to analyze patterns of peptides generated by partial digestion of proteins and reverse phase HPLC has been used to analyze peptides generated from complete enzymatic digests.

Reverse phase HPLC gives excellent fractionation of small peptides but, in our experience, does not work well for the separation of peptide species 50 amino acids in length or longer. In addition, HPLC requires the researcher to have access to expensive equipment which is not readily available to all researchers. In contrast, SDS-PAGE has been widely used for fractionation of peptides of 90-100 amino acids and larger, such as those generated from the partial digestion of a target protein or by chemical digestion methods (such as cyanogen bromide) that generate relatively large fragments.

Recently introduced modifications to SDS-PAGE allow the resolution of peptides as small as 20 amino acids in length. Such peptide separation gels, first described by Schägger and von Jagow (6), have been refined and adapted for use in a minigel format by Promega.

The Probe-Design™ peptide separation system, described in Section IV (pg. 256), combines cyanogen bromide cleavage reagents and peptide separation gels in a convenient kit which allows the preparation of N-terminal blocked proteins for sequencing.

C. Protein Sequence Analysis

Protein sequence analysis is useful for designing oligonucleotide probes employed in cloning the gene encoding a target protein (Section IV, pg. 256). Protein sequence data also provides protein structural information that is difficult to obtain in any other way. For example, sites of phosphorylation or antibody binding can be mapped in a straightforward way to specific peptide fragments, which then can be identified unambiguously by protein sequencing. Obtaining this information by other approaches, such as site-directed mutagenesis, is much more time consuming.

Many proteins have a chemically blocked amino terminus, rendering them refractile to direct sequence analysis. As a result, it often is necessary to fragment a target protein using proteases or chemical cleavage reagents before sequence information can be obtained. The advantages and disadvantages of these two cleavage approaches are discussed in Sections D and E, below.

When a protein is to be cleaved for sequence analysis, a complete digestion is generally better than a partial digestion. Partial digestion of a protein generates a larger number of different peptide fragments than complete digestion, resulting in a lower molar amount of each peptide. For example, if 5µg of a 50kDa protein (100pmoles) is partially digested and five fragments contain the bulk of a region to be submitted for sequence analysis, none of the fragments is likely to contain more than about 20pmoles of peptide. A complete digest, however, should generate 100pmoles of each fragment. Much more sequence information can usually be obtained from a 100pmole sample than a 20pmole sample.

The products of complete digests may be resolved by HPLC or, for fragments as small as 20 amino acids, on peptide separation gels. In cases where much more sample is available, a partial digestion may be desirable simply because the larger fragments generated may be easily resolved by normal SDS-PAGE.

I. General Considerations for Protein Analysis

(continued)

Table 1. Common Methods for Chemical Cleavage of Proteins.

Reagent	Target sequence	Conditions	Reference
Cyanogen Bromide	Met-X	70% formic acid, room temperature, 5hrs.	7
Partial Acid Cleavage	Asp-Pro	70% formic acid, 37°C, 24-72hrs.	8
Hydroxylamine	Asn-Gly	2M hydroxylamine pH 9-10, 37°C, 1-4hrs.	9

D. Chemical Cleavage of Proteins

A variety of chemical reagents have been developed for the cleavage of proteins at specific sequences. These reagents are very effective for cleavage of the polypeptide chain and, with the exception of partial acid proteolysis at Asp-Pro bonds in formic acid, they are independent of the sequence context of the sensitive site. These reagents operate best on denatured protein species; the reaction conditions employed, however, usually guarantee that the target protein will be denatured during digestion. A listing of some of the more common and reliable chemical cleavage techniques is given in Table 1.

The use of the reagents listed above is sometimes made more difficult due to the state of the sample before digestion. For example, cyanogen bromide (CNBr) digestion of a protein sometimes fails because the target protein has been oxidized prior to digestion. Oxidation can result in the transformation of methionine residues into methionine sulfoxide or sulfone residues. Such modified forms of methionine are not attacked by CNBr, and thus the regions of the protein that contain such modified sites will not be cleaved by this reagent. In general, however, chemical cleavage methods are more reliable than protease treatments and give a more complete digestion of a protein sample.

One additional difficulty in the use of chemical cleavage reagents is that the relatively large peptide species generated are usually insoluble in commonly used buffers. This makes the fractionation of such fragments by normal column chromatographic methods impossible. These fragments may easily be separated by SDS-PAGE, however, if they are coated with detergent. This procedure is described in detail in Section IV (pg. 256).

E. Enzymatic Cleavage of Proteins

The cleavage of a target protein for sequence analysis is most commonly done with proteases, because the peptides generated are usually soluble in the solvents used in column fractionations. The proteases typically used in sequence analysis are highly specific, allowing peptides to be generated by cleavage of proteins at predictable, defined sites. Promega supplies highly purified sequencing grade proteases which are tested to ensure the absence of non-specific cleavage activities. Specific proteases most commonly are used for complete digestion of a target protein, producing fragments averaging about 20 amino acids in length. These fragments are well resolved by reverse phase HPLC or peptide separation gels.

The difference in specificity of specific and general proteases is defined by the number of sequences that the protease will attack under normal conditions (Table 2). For example, endoproteinase Glu-C will cleave protein substrates only at glutamic acid residues under normal conditions and is considered to be a specific protease, while alkaline protease will cleave the same protein primarily at aromatic and large hydrophobic amino acids (Trp, Tyr, Phe, Leu, Val, and Ile) under normal conditions and is considered to be a general protease.

General proteases often are used for removal of protein in solutions of other types of biomolecules such as DNA. For these applications, general proteases are preferred since they usually can begin digestion of the target protein before it is denatured. Some applications, such as protein fingerprinting, use both types of proteases. A general protocol for complete protease digestions is provided on pg. 244.

I. General Considerations for Protein Analysis

(continued)

Table 2. Cleavage Specificities of Proteases Supplied by Promega.

Protease	Cleavage Specificity	Notes
Specific Proteases		
Clostripain (Endoproteinase Arg-C)	- Arg▼ X-	Clostripain requires the presence of a sulfhydryl reagent such as DTT for activity.
Endoproteinase Glu-C (*S. aureus* V8 protease)	- Glu▼ X- (- Asp▼ X-)	The specificity of endoproteinase Glu-C depends on the buffer used for digestion. It specifically cleaves at the carboxylic side of glutamic acid in 50mM ammonium bicarbonate, pH 7.8, or 50mM ammonium acetate, pH 4.0. In 50mM sodium phosphate, pH 7.8, this protease cleaves after both glutamic acid and aspartic acid (10,11).
Endoproteinase Lys-C	- Lys▼ X-	Promega's endoproteinase Lys-C works well in the presence of up to 2% SDS.
Modified Trypsin	- Arg▼ X- - Lys▼ X-	When proline is at the carboxylic side of arginine or lysine, the peptide bond is almost completely resistant to cleavage by trypsin. Cleavage may also be considerably reduced when acidic residues are present on either side of a potentially susceptible bond (10).
General Proteases		
Alkaline Protease (Subtilisin Carlsberg)	- Trp▼ X- - Tyr▼ X- - Phe▼ X- - Leu▼ X- - Val▼ X- - Ile▼ X-	Cleavage occurs at the carboxylic sides of aromatic and large hydrophobic amino acids.
Proteinase K	Broad specificity	Cleavage occurs preferentially at the carboxylic sides of aliphatic and aromatic amino acids (12).

In general, proteins require denaturation and disulfide bond cleavage before enzymatic digestion can go to completion (10). Denatured proteins often are insoluble in aqueous solutions in the absence of denaturants. Promega's sequencing grade proteases are active in the low concentrations of denaturants needed to preserve solubility of most denatured proteins. Some denatured proteins, however, will be insoluble even in relatively high concentrations of denaturant. Such samples should be dispersed as finely as possible before digestion is attempted.

The amount of protease added should allow good digestion of the target protein yet be low enough that it will not generate too high a concentration of fragments from the protease. Most digestions work well with a protease:protein ratio of 1:100 to 1:25 (w/w), but a 1:10 ratio may be used for proteins more resistant to hydrolysis. When high protease:protein ratios are used, an enzyme blank can be prepared to allow identification of peptides generated by self-digestion of the protease. Polypropylene microcentrifuge tubes should be used for digestions, since peptides will bind with high affinity to glass surfaces.

Protein Analysis

I. General Considerations for Protein Analysis

(continued)

General Protease Digestion Protocol

Reagents Needed

- denaturing solution
- 50mM ammonium bicarbonate, pH 7.8

1. Dry the protein sample (up to 100µg of protein) in a microcentrifuge tube from a low salt (<50mM) solution.
2. Resuspend the dried sample to a 1mg/ml concentration in denaturing solution (below) and heat at 37°C for 1 hour to denature and reduce the protein. A 1mg/ml protein concen- tration is optimal, but no less than 20µl of solution should be used to minimize evaporative losses, even if this results in a more dilute solution.
3. Dilute the solution 4- to 10-fold with 50mM ammonium bicarbonate, pH 7.8, or another buffer recommended for the protease to be used. If the target protein precipitates at this point, disperse the precipitate by pipetting up and down in a plastic tip.
4. Add protease to the desired concentration (typically 1:100 to 1:10 protease:protein ratio) and incubate overnight at 37°C. Also, perform a control digestion which contains protease but no target protein.
5. If desired, a second aliquot of protease, equivalent to that added above, may be added the next day. Incubate the sample at 37°C for 1 hour more.
6. The results of the digestion can be analyzed by HPLC or SDS-PAGE separation of the peptide fragments. A control digestion can be used to determine if any of the fragments obtained may have resulted from self digestion of the protease added.

Solution Composition

Denaturing solution:

50mM	ammonium bicarbonate, pH 7.8
8M	urea
0.05%	SDS
2mM	DTT

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F. Applications of Protein Fingerprint Analysis

Protein fingerprinting is a powerful tool for determining the structural relatedness of proteins and for analyzing a variety of protein modifications. In this procedure, target proteins are subjected to partial proteolytic digestion followed by SDS-PAGE separation of the peptides generated (13). The resultant patterns of fragments, or "fingerprints", are characteristic of the particular protein substrates and proteases used for cleavage. Promega's protein fingerprinting system, described in Section III (pg. 250), offers a convenient and economical means to perform this technique.

Protein fingerprinting may be used to analyze the relatedness of many categories of proteins, including "natural" and cloned gene products, proteins showing cross-reactivity with a common antibody, and proteins having various covalent post-translational modifications. This approach can be used, for example, to compare the incorporation of ^{32}P into a target protein phosphorylated *in vivo* and *in vitro.* Autoradiography can be used to determine the pattern of ^{32}P-labeled fragments after partial proteolysis of the two protein samples. A difference in the pattern of labeled fragments indicates a difference in the locations of modifications. However, if the fingerprint pattern of the two samples appears identical, it does not guarantee that identical residues are modified. Thus, while protein fingerprinting can quickly and economically provide strong positive evidence for differences, it can not supplant direct protein sequence analysis for prease determination of the sites of modifications.

Protein fingerprinting also can be used in conjunction with Western blotting (Section V, pg. 262) to quickly compare the peptide fragments bound by different monoclonal antibodies. In these cases, the antibodies used must be able to bind to the denatured protein, a condition usually satisfied by knowing that the antibody is reactive on Western blots. If two antibodies produce an identical pattern of crossreactive bands, their binding sites are close together. If the pattern of antibody-reactive fragments diverges at a part of the gel containing large protein fragments, the binding sites are relatively far apart in the primary sequence of the target protein (14). While fingerprinting does not allow precise mapping of antibody binding sites, it is useful as a rapid and sensitive method to look for large changes.

II. Visualization of Gel Bands During Electrophoresis using the ChromaPhor™ System

A. General Considerations

The ChromaPhor™ gel system employs a special gel/buffer formulation combined with Promega's novel ChromaPhor™ green protein stain* to visualize protein bands while the gel is running. There are three levels of sensitivity outlined in the protocol which should be used depending on the application (Table 3). As little as 1µg of protein per band can be visualized as the gel is running (using 0.75mm thick minigels). This level of sensitivity is ideal for preparative electrophoresis, where large amounts of proteins are typically loaded on a gel. Because the interaction between the dye and protein bands does not "lock" the protein into the gel or alter it chemically, the protein can be used in further analysis. In addition, the ability to visualize protein bands as they separate offers several advantages:

- The quality of the run can be checked continuously.
- The resolution of specific bands can be optimized as the gel is running.
- Fractions suitable for pooling can be identified when analyzing chromatography results.
- Gel bands can be instantly localized, without fixation in the gel.

Enhancement of Detection Sensitivity

A rapid enhancement protocol (2-3 minutes) can be used to increase the sensitivity of detection to 100-200ng of protein per band (see Section C, pg. 248).

This method is gentler than most staining methods, and will generally give better recovery of the protein than Coomassie or silver staining methods. In the case of an analytical electrophoretic run, where further sensitivity may be required, a full enhancement/destaining protocol (60 minutes) is provided. This method is faster than conventional destaining methods, but it does expose the bands to conditions similar to other staining methods which may alter the proteins and therefore is not recommended for preparative procedures.

It is important to note that the ChromaPhor gel system employs an SDS concentration in all gel components lower than that used in standard procedures. The use of gel components other than those provided may result in reduced sensitivity. Samples containing high levels of detergent should be TCA-precipitated before analysis to prevent interference with in-run staining. However, protein detection after full enhancement (Table 3) does not seem to be affected by the presence of SDS or other detergents in the sample. Almost all proteins exhibit identical mobility in the ChromaPhor system and standard SDS-PAGE systems, but some mobility differences may be seen with a small number of proteins.

In general, this system should be suitable for most of the same applications as Coomassie Blue. A protocol is provided in Section D, pg. 248 for elution of proteins from a gel slice. Many other potential applications may be realized with this novel protein visualization system. Please contact our Technical Services department for more information.

*Patent Pending

Table 3. Detection Sensitivity of the ChromaPhor System in Minigels.

Evaluation of detection sensitivity was based on visualization of protein bands of Promega's mid-range protein molecular weight markers in a 0.75mm thick minigel.

Visualization Method	Detection Sensitivity	Post-Run Treatment
Visualization during run, preparative or analytical	1µg	None
Rapid enhancement, preparative or analytical	100-200ng	2-3 minutes (5% acetic acid)
Full enhancement/destaining, analytical	<100ng	60 minutes (30% methanol/ 5% acetic acid)

II. Visualization of Gel Bands During Electrophoresis using the ChromaPhor™ System

(continued)

B. ChromaPhor Gel System Protocol

Reagents to be Supplied by the User

- 10% (w/v) ammonium persulfate
- TEMED (N,N,N′,N′-tetramethylethylenediamine)
- running buffer (pg. 249)
- trichloroacetic acid (optional)
- acetone (optional)

Select the acrylamide percentage appropriate for the size of proteins to be separated (see Table 4). The recommended ranges overlap because proteins substantially different in size may need to be analyzed together, as in the case of a small protease inhibitor noncovalently bound to a much larger protease. If you plan to recover a particular protein from the gel, use the lowest gel percentage that is effective in fractionating the sample.

Table 4. Recommended Acrylamide Gel Percentages.

Recommended % Acrylamide	Protein Size Range
8%	40-200kDa
10%	21-100kDa
12%	10-40kDa

Table 5. Preparation of ChromaPhor System Gels.

Resolving Gel Components	Volume Added for Desired Acrylamide Percentage: 8%	10%	12%
lower gel 4X buffer (supplied)	2.5ml	2.5ml	2.5ml
deionized H_2O	5.4ml	4.9ml	4.4ml
acrylamide stock solution (supplied)	2.0ml	2.5ml	3.0ml
10% (w/v) ammonium persulfate	50µl	50µl	50µl
TEMED	5µl	5µl	5µl

Stacking Gel Components	Volume Added
upper gel 4X buffer (supplied)	2.5ml
deionized H_2O	6.6ml
acrylamide stock solution (supplied)	0.8ml
10% (w/v) ammonium persulfate	100µl
TEMED	10µl

1. Assemble the gel apparatus using the manufacturer's instructions. If possible, insert a thin sheet of white plastic behind the gel plates so that the proteins can be more easily seen during the run.

2. Prepare the gel solution using the reagents supplied and the appropriate recipe from Table 5. The volumes given are sufficient to pour two 7 x 9cm minigels, 0.75-1.0mm thick. **Caution:** Acrylamide is a neurotoxin and should be considered hazardous. Wear gloves and use care to avoid acrylamide ingestion or contact with skin. Add the ammonium persulfate and TEMED just prior to pouring the gel, as these reagents catalyze the polymerization of acrylamide. For best results, always use freshly prepared ammonium persulfate solution.

II. Visualization of Gel Bands During Electrophoresis using the ChromaPhor™ System

(continued)

3. Pour the resolving gel mix into the assembled gel plates, leaving sufficient space at the top for the stacking gel to be added later. Gently overlay the gels with 200-400µl of deionized H_2O and allow the gel to polymerize for 15-30 minutes.

4. After polymerization, remove the overlay and rinse the surface of the resolving gel with water to remove any unpolymerized acrylamide. Rinse one more time with 100µl of upper gel buffer diluted to 1X.

5. Mix the upper gel components listed in Table 5. Fill the remaining space in the gel apparatus with the upper gel solution and insert the comb immediately. At least 1cm of upper gel should be present between the bottom of the loading wells and the resolving gel.

6. After the upper gel has polymerized, remove the comb, rinse the wells with water to remove unpolymerized acrylamide, and fill the gel reservoirs with running buffer (pg. 249). The running buffer used with the ChromaPhor system gels contains a lower than usual SDS concentration. Note the approximate volume of running buffer added to the upper reservoir.

Sample Preparation

7. Mix each sample with an equal volume of the loading buffer provided and heat to ≥95°C for 2-5 minutes to denature the proteins. Sample volumes should be ≤20µl for optimal in-run staining.

   If the sample is too dilute to use directly or if it contains a high detergent concentration (>1%), precipitate the sample by the addition of trichloroacetic acid (TCA) to 10% (w/v). Place the sample at 4°C for 5 minutes, collect the pellet by centrifugation and wash it with cold acetone. Resuspend the resultant protein pellet in an appropriate volume of loading buffer, prepared by 1:1 dilution of the 2X loading buffer supplied. **Note:** Do not use a standard SDS-PAGE 2X loading buffer; the ChromaPhor system utilizes a reduced SDS concentration in all gel buffers.

   Denature the sample by heating for 5 minutes at 95°C.

8. Load the samples on the gel and then dilute the green ChromaPhor stain 1:1,000 into the upper reservoir buffer (e.g., 0.2ml stain/200ml running buffer).

9. Run the gel according to the recommendations of the apparatus manufacturer. The green stain will act as a tracking dye during electrophoresis. Stop the run when the proteins of interest are adequately resolved or when the green dye runs off the bottom of the gel. Protein bands may be cut from the gel and eluted as described in Section D (pg. 248).

   Note: The ChromaPhor system is not recommended for in-run staining of large format (16cm x 16cm) SDS gels, as the staining becomes excessively faint in the lower portion of the gel.

II. Visualization of Gel Bands During Electrophoresis using the ChromaPhor™ System

(continued)

C. Enhancement of Protein Detection

Reagents Needed

- 5% (v/v) acetic acid (for rapid enhancement)
- 30% (v/v) methanol, 5% (v/v) acetic acid (for full enhancement)

Two alternative methods are provided for enhancement of protein detection after electrophoresis (see Table 3, pg. 245). The first method is extremely rapid and minimizes exposure of protein bands to harsh conditions, while the second method provides the best possible sensitivity of detection. If it becomes apparent that additional detection sensitivity is needed, bands visualized with the ChromaPhor system can be subsequently silver stained (4).

Rapid Enhancement Protocol for Preparative SDS-PAGE

1. Place the gel in a solution of 5% (v/v) acetic acid for 2-3 minutes. The gel will develop a light green background color and protein bands should be visible in the range of 100-200ng per band in a 0.75mm minigel. The gel may be stored in this solution.

Full Enhancement Destaining Protocol for Analytical SDS-PAGE

1. Place the gel in a solution of 30% (v/v) methanol, 5% (v/v) acetic acid and agitate gently at room temperature for 60 minutes. As little as 60ng protein per band should be detectable in a 0.75mm minigel against a nearly clear background.

Notes:

1. Gels stained by either method can be dried using Promega's gel drying frames and film. Dried gels can be placed directly on an overhead projector for presentations. A good quality photocopy can be made if a white sheet is placed behind the gel.

2. Suggestions for photographing dried gels:

   a. Black and White - Polaroid Type 665 film with red filter #25A or no filter

   b. Color - Kodak Gold 100

D. Protein Recovery from Gels

Reagents Needed

- gel elution buffer (pg. 249)
- 80% acetone (optional)

Ideally, the protein to be eluted from the gel should be loaded at ≥1µg per lane so that no post-run enhancement will be needed to visualize the band. If staining enhancement is necessary, such as when monitoring for possible contaminating bands, we recommend using the 2-3 minute rapid enhancement method preferentially in order to minimize the exposure of the protein to acid. The protein band(s) of interest may be eluted by passive diffusion (described below) or by using a commercial elution device.

1. Excise the band(s) of interest. Placing the gel on a light box can be helpful in locating the bands.

2. Mince the gel slice as finely as possible in a microcentrifuge tube. This is conveniently performed with a gel mincing device available from Kontes® Corporation.

3. Add 100-200µl of gel elution buffer (pg. 249) and incubate at 37°C for at least one hour.

4. Pellet the acrylamide fragments by centrifugation for 2 minutes in a microcentrifuge. Transfer the supernatant to another microcentrifuge tube.

5. Wash the acrylamide fragments with 100µl of gel elution buffer, pellet the fragments and pool the two supernatants.

6. The ammonium bicarbonate solution containing the eluted protein can be concentrated by drying in a Speed-Vac® vacuum concentrator.

7. If desired, the dye and residual SDS bound to the protein precipitate can be removed. This can be done by adding 100µl of cold (–70°C) 80% acetone in the tube, vortexing for 30 seconds and placing the solution in a dry ice/ethanol bath for 3-5 minutes. Spin in a microcentrifuge for 2 minutes at 4°C. Remove and discard the supernatant. Redry the pellet.

We routinely recover about 60% of loaded protein using this method.

II. Visualization of Gel Bands During Electrophoresis using the ChromaPhor™ System

(continued)

Composition of Solutions

Running buffer, pH 8.3:

25mM	Tris-base
192mM	glycine
0.035%	SDS

Gel elution buffer:

50mM	ammonium bicarbonate, pH 7.8
0.1%	SDS

E. Troubleshooting

Problem	Possible Cause	Comments
Gel bands not visible during electrophoresis.	Protein concentration too low.	Concentrate the sample using TCA or other methods.
	Excessive detergent in sample.	Remove detergent by precipitation of the sample with TCA or other methods.
	Incorrect concentration of ChromaPhor™ green stain.	Verify that stain was diluted 1:1,000 into the upper reservoir buffer.
	Gel too thick.	Visualization during gel run functions best with gels <1.0mm thick.
	Insufficient time for dye to interact with proteins.	Decrease amperage or voltage during electrophoresis.

Protein Analysis

III. Protein Fingerprinting

A. General Considerations

Analysis of the protein composition in a mixed sample is most often accomplished by SDS polyacrylamide gel electrophoresis using electrophoretic mobility as the indicator of a protein's identity. However, the relatedness or identity of two or more proteins can not be deduced from their mobility in SDS-PAGE gels alone. Proteins showing identical mobilities can have little or no sequence homology, while proteins of differing mobilities may share regions of high identity but differ as a result of proteolytic processing or covalent modification. For these reasons, additional analytical methods are useful.

Promega's protein fingerprinting system, based on the method of Cleveland (13), can serve as a powerful tool for determining if proteins are related. This method utilizes partial proteolytic digestion of proteins followed by SDS-PAGE separation of peptide fragments to generate a "fingerprint" which is characteristic of the particular protein substrate and the protease used for cleavage. The protein substrate may be in the form of a homogeneous solution, electroeluted from gels, or isolated as a gel slice from a standard SDS-PAGE slab gel. The protein fingerprint is generated in a second SDS-PAGE gel of higher acrylamide concentration where proteolytic digestion and electrophoretic separation of stable partially digested peptides occur simultaneously. Peptide fingerprints of a given protein substrate are highly reproducible and are independent of the substrate form (purified, electroeluted, or gel slice). The ability to apply this method to protein in a gel slice avoids the problematic methods of electroelution which can be both time-consuming and result in poor recoveries of protein. As little as 10µg of protein substrate is required for fingerprinting using the Cleveland method. Analysis generally involves 3-5 partial digestion reactions in adjacent lanes of an SDS gel. Each lane contains a standard amount (≥1µg) of the substrate protein with increasing protease concentrations (Figure 1). This ensures that the optimal protease concentration for comparison of the substrate proteins will be used. Two or more protein fingerprints can be performed on a single slab gel, allowing the researcher to make direct unambiguous comparisons. Additional analytical power is gained by performing fingerprinting with a variety of proteases because each protease generates a distinct fingerprint from a single substrate.

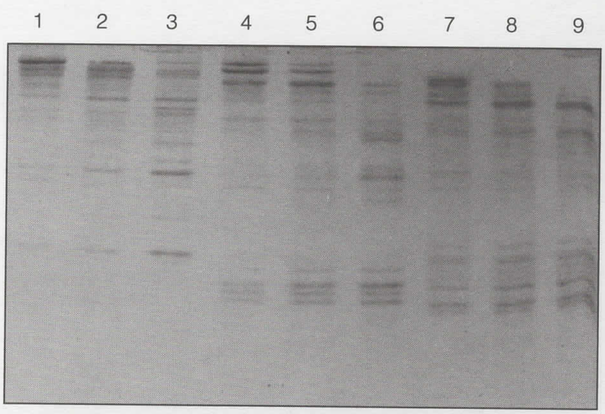

Figure 1. Phosphorylase B subjected to partial proteolysis with three different proteases. Individual phosphorylase B samples were digested with different amounts of the following proteinases: endoproteinase Lys-C, endoproteinase Glu-C, and alkaline protease. Lane 1, 1:200 endoproteinase Lys-C; lane 2, 1:100 endoproteinase Lys-C; lane 3, 1:20 endoproteinase Lys-C; lane 4, 1:200 endoproteinase Glu-C; lane 5, 1:100 endoproteinase Glu-C; lane 6, 1:20 endoproteinase Glu-C; lane 7, 1:4,000 alkaline protease; lane 8, 1:2,000 alkaline protease; lane 9, 1:1,000 alkaline protease.

III. Protein Fingerprinting

(continued)

The exact pattern of a protein fingerprint is a result of the substrate specificity of the protease used and also is dependent on the number of susceptible sites in the protein substrate. For this reason, the degree of similarity in the peptide fingerprints of two proteins correlates with the degree of common evolutionary and/or genetic origin. A mathematical model for quantitating the similarity between two proteins has been suggested by Calvert and Gratzer (15). This method is based on analysis of the number of peptides showing identical mobilities.

B. Primary Gel Electrophoresis

Reagents to be Supplied by the User

Instructions for preparing these reagents are provided on pg. 255.

- Coomassie stain
- destaining solution
- running buffer

We recommend that molecular weight markers be stored in several aliquots to avoid repeated freeze-thaw cycles and to prevent accidental contamination or degradation of the entire stock.

Protocol

1. Perform gel electrophoresis of a non-homogeneous mixture (i.e., cell extract, column fractions, etc.) using the discontinuous system described by Laemmli (16).

   Resolving minigels should be cast allowing room for a 2cm stacking gel. Fingerprint minigels (for resolving protease digestion products) require a 2.5cm stack. For casting of 16 x 18cm gels, the volumes in Tables 6 and 7 should be increased 4-fold, allowing for a 3cm stacking gel (4cm stack for fingerprint gels). Gel thickness should be limited to 0.75-1.0mm for the initial gel. This facilitates the transfer of gel slices into a second SDS-PAGE fingerprinting gel (1.0-1.5mm thickness).

   Mid-range protein molecular weight markers are included with the protein fingerprinting kit for convenience in identifying the protein bands of interest. A clear banding pattern is obtained with 1-2µl of the stock suspension, which is provided in a sample buffer containing SDS, reductant, and glycerol. For convenience in loading, this stock solution may be diluted 10-fold with loading buffer (pg. 255). To ensure complete denaturation of the markers, heat them for 5 minutes at 95°C before loading the gel.

2. Following electrophoresis, stain the gel with Coomassie stain (pg. 255) for 10 minutes. Destaining is done by immersion in destaining solution (pg. 255). Limit the destaining step to 60 minutes or less. The use of a rotary shaker is recommended for both staining and destaining.

3. After destaining, rinse the gel in distilled water, place the gel on a glass plate and illuminate over a light box. Locate the target protein bands and excise them with a razor blade. Equilibrate the gel slices in running buffer (pg. 255) for 30-60 minutes at room temperature. Allow 3-5ml of buffer per gel slice.

Notes:

1. Staining and destaining times are critical to both the recovery and efficiency of digestion of gel-isolated protein in subsequent fingerprint gels. Extended staining and/or destaining can cause protein to be permanently fixed within the gel matrix and can cause acid cleavage of some proteins. Such problems may be avoided by running gels with Promega's ChromaPhor™ protein visualization system, which allows bands to be seen as the gel runs.

2. Residual acetic acid will cause streaking on fingerprint gels. Be sure to allow at least 30 minutes for equilibration. Gel slices can be stored at –20°C. However, best results are obtained when the gel slices are loaded onto fingerprint gels immediately after equilibration.

III. Protein Fingerprinting

(continued)

C. Fingerprinting of Gel-Isolated Proteins

1. Fingerprinting gels are prepared the same as standard Laemmli gels with the exception that higher polyacrylamide concentrations are used for both the stacking and resolving gels. Prepare the stacking gels using a 4.5% acrylamide concentration. Prepare resolving gels of 15% acrylamide for protein substrates greater than 30kDa, and 20% acrylamide for substrates less than 30kDa. The gel thickness should be 1-1.5mm to accommodate gel slice loading.

2. Trim the gel slices (if necessary) to the approximate width of the wells of the finger-printing gel. Flood the wells with running buffer, then load gel slices using the provided tool. Position the slices horizontally to contact the well bottom. Care should be taken to eliminate any air bubbles trapped under the gel slices. Fill the spaces around the gel slices by overlaying with 5μl of gel slice overlay solution (pg. 255).

3. Resuspend each tube of lyophilized protease in 100μl of the appropriate storage buffer (0.2mg/ml final concentration). Store the unused portion frozen in 20μl aliquots.

   Serially dilute the protease selected for digestion in protease dilutent solution (pg. 255) such that a 5μl overlay represents a protease:protein ratio of between 0.025% and 10%(w/w). To identify bands contributed by the protease rather than the target protein, it is advisable to include a control lane containing protease only at a concentration equalling that of the highest amount used for digestion. Protease ratios of 0.025%-2% are recommended for alkaline protease while ratios of 0.5%-10% are suggested for endoproteinase Lys-C and Glu-C.

   For example, if each lane contains a gel slice with an estimated 2.5μg of the provided control protein substrate, the following dilution series could be used for endoproteinase Lys-C or Glu-C:

Gel Lane	Protease Concentration (mg/ml)	Volume Added (μl)	% Protease (w/w)
1	0.0	5	0.0
2	0.0025	5	0.5
3	0.0050	5	1.0
4	0.0125	5	2.5
5	0.0250	5	5.0
6	0.0500	5	10.0

III. Protein Fingerprinting

(continued)

4. Electrophoretic conditions are dependent upon the gel size and electrophoretic chamber used. The following conditions are recommended for the most commonly used electrophoresis systems cast with 1.0mm thick gels:
   - Biorad Miniprotean® II (7 X 9cm gels): Set the initial voltage to 100V for 15-20 minutes to stack proteins at the interface between the stacking and resolving gels. Interrupt the voltage for 15-30 minutes to allow for digestion. Finally, apply 200V until the dye front reaches the gel bottom (approximately 35 minutes).
   - Hoeffer vertical slab unit (16 X 18cm gels): Set the initial voltage to 150V for 45-60 minutes to stack proteins at the interface between the stacking and resolving gels. Interrupt the voltage for 15-30 minutes to allow for digestion. Finally, apply 250V until the dye front reaches the gel bottom.

5. Following electrophoresis, peptide fingerprint patterns can be visualized using Coomassie staining (see Step B.2, above) or by alternate staining methods such as silver-staining (4). For Coomassie staining, a minimum of 2.5µg of protein substrate should be loaded per lane. As little as 0.5µg of substrate may suffice if silver-staining is used to detect the proteolysis products. This option is particularly attractive when limited quantities of substrate are available. A general rule for protein fingerprinting is: the larger the protein, the greater the mass of protein required for detection by staining.

Notes:

1. The efficiency of digestion in fingerprint gels can be increased for a given amount of protease by: preparing longer stacking gels, using lower voltages for stacking, or by extending the power interruption step during electrophoresis.

2. These are general application guidelines, however it should be remembered that each protein is unique in its behavior as a substrate for proteolytic cleavage. Initial experiments should include a broad range of protease dilutions to optimize fingerprint results.

D. Fingerprinting of Purified or Electroeluted Proteins

1. For digestion of soluble samples, dilute the protein substrate in gel slice overlay solution (pg. 255) to an appropriate concentration and heat-denature by incubating at 95°C for 5 minutes. After cooling, place equal aliquots (5-10µl) in separate microcentrifuge tubes.

2. Serially dilute the protease selected as described in Step C.4, above. Mix 5µl of each protease dilution with an aliquot of protein substrate and immediately load the mixture into the wells of a fingerprint gel.

E. Interpretation of Results

Partial proteolytic fingerprints are remarkably reproducible and allow for side-by-side comparisons of the degree of relatedness between two of more proteins on the same gel. Calvert and Gratzer (15) have described a method for calculating the degree of relatedness of the two proteins, based on the number of peptide bands showing identical mobility in comparative fingerprints.

Supplemental data may be gathered by additional fingerprint comparisons using additional proteases for digestion. The amino acid composition of the protein substrate is the determining factor for which protease will be most efficient for fingerprinting analysis. Endoproteinase Lys-C cleaves at the carboxylic side of lysine residues while endoproteinase Glu-C cleaves at the carboxylic side of glutamic acid residues. Alkaline protease has a more general catalytic activity, cleaving preferentially at aromatic residues, then at large hydrophobic amino acids, and finally at alternative sites when used at very high concentrations.

III. Protein Fingerprinting

(continued)

Table 6. Formulation for SDS-Polyacrylamide Resolving Gels. (Recipes are sufficient for the preparation of two 7 x 9cm gels.)

	Volume for Different Percentages of Acrylamide				
Component	8%	10%	12%	15%	20%
Resolving gel 4X buffer (ml)	2.5	2.5	2.5	2.5	2.5
Water (ml)	4.8	4.2	3.5	2.5	0.8
Acrylamide (ml)	2.7	3.3	4.0	5.0	6.7
10% APS* (ml)	0.05	0.05	0.05	0.05	0.05
TEMED** (µl)	5.0	5.0	5.0	5.0	5.0

*ammonium persulfate
**N,N,N′N′-tetramethylethylenediamine

Table 7. Formulation of Stacking Gel.

	Volume for Different Percentages of Acrylamide	
Component	3%	4.5%
Stacking gel 4X buffer (ml)	1.25	1.25
Water (ml)	3.2	3.0
Acrylamide (ml)	0.5	0.75
10% APS (ml)	0.1	0.1
TEMED (µl)	10.0	10.0

Composition of Solutions

Acrylamide stock (30:0.8):

30%	acrylamide
0.8%	bisacrylamide

Stacking gel 4X buffer:

0.5M	Tris-HCl
0.4%	SDS, pH 6.8

Resolving gel 4X buffer:

1.5M	Tris-HCl, pH 8.8
0.4%	SDS

Running buffer 4X stock:

0.1M	Tris, pH 8.3
0.77M	glycine

Running 1X buffer:

1X	running buffer stock
0.1%	SDS

Loading 2X buffer:

25%	stacking gel 4X buffer
2%	SDS
5%	β-mercaptoethanol
20%	glycerol
0.0025%	bromophenol blue

Coomassie stain:

25%	isopropanol
10%	glacial acetic acid
0.25%	Coomassie brilliant blue (R250)

Destaining solution:

7%	glacial acetic acid

Protease diluent 5X solution:

0.63M	Tris, pH 6.8
0.5%	SDS
50%	glycerol
0.025%	bromophenol blue

Gel slice overlay solution:

0.125M	Tris, pH 6.8
0.1%	SDS
3%	β-mercaptoethanol
20%	glycerol
0.005%	bromophenol blue

Protein Analysis

IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

A. General Considerations

When a molecular biologist has identified a protein that is important to the biological system under study, he or she has few options in obtaining probes that can be used to screen a library for a clone of the gene that encodes the protein. If a sufficient amount of the protein can be isolated, an antibody to the protein may be generated to screen expression libraries. If the gene is very similar to another gene whose sequence has been determined, probes based on the known sequence can be used. However, if very little is known about the protein of interest, the best method would be to design probes based on portions of the protein sequence of the target protein. Unfortunately, proteins are often chemically blocked at their N-termini and can not be directly sequenced. Preparation of such proteins for sequencing, particularly where only a few micrograms of the protein are available, usually requires sophisticated protein chemistry techniques as well as equipment (such as microbore reverse phase HPLC) that may not be readily available.

The Probe-Design peptide separation system was developed to simplify the generation of internal sequence from proteins using techniques that do not require specialized protein separation strategies nor equipment. Using this system, 250pmoles of a gel band or 100pmoles of a pure protein in solution may be cleaved into peptide fragments, separated, and prepared for direct protein sequencing. Sequence data obtained from selected peptides may then be used to design an oligonucleotide probe capable of hybridizing to the gene encoding the protein of interest. The Probe-Design system may also be used to localize sites of post-translational protein modifications to individual peptide fragments.

To use the Probe-Design peptide separation system, the target protein is purified by SDS polyacrylamide gel electrophoresis, transferred to PVDF blotting membrane (supplied), stained, and the desired band is cut out. Complete cyanogen bromide (CNBr) cleavage at methionine residues is performed on the immobilized band (or on purified target protein in solution) and the small peptide fragments thus generated are separated by electrophoresis on high resolution polyacrylamide minigels. The use of these specially formulated gels eliminates the need to use HPLC and allows the use of less starting material than is needed for a standard-bore HPLC peptide separation. The separated peptides are transferred to a PVDF sequencing membrane (supplied) and selected bands are cut out, ready for direct sequence analysis*.

The system includes the specialized reagents and membranes necessary to perform cyanogen bromide cleavage of approximately five target proteins as well as the reagents and membranes needed for gel separation and immobilization of the resultant peptide fragments. A positive control protein and low-range protein standards are included to allow the user to monitor each step of the procedure. Protective agents are employed at several steps to prevent chemical N-terminal blockage of the peptides generated. To further increase the yield of peptides, two types of PVDF membrane are included with the Probe-Design system. Digestion of target proteins on PVDF blotting membrane leads to better recovery of eluted peptides, while electroblotting of these small peptides to PVDF sequencing membrane results in significantly higher yields when this membrane is used as the solid support for protein sequence analysis.

* This basic methodology was independently developed at Promega and in Dr. Daniel Crimmins' laboratory (17).

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IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

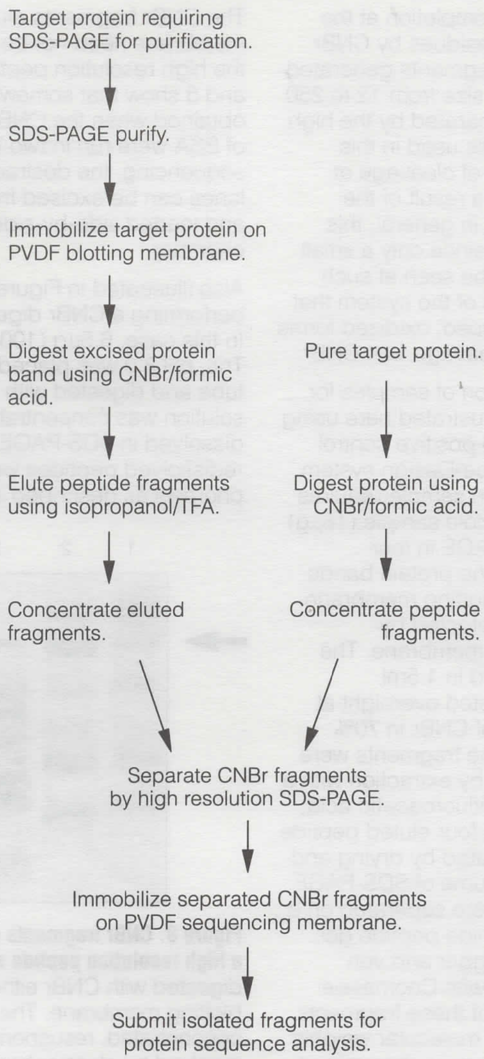

Figure 2. Schematic diagram of the Probe-Design peptide separation system procedure.

Overview of the Probe-Design System Procedure

When possible, the starting material for the Probe-Design procedure should be a pure protein in solution, obtained either by classical protein purification methods or by electroelution from gels. In this case, CNBr cleavage of the target protein is performed in solution in a dilute buffer not containing reductants such as DTT (which react with CNBr). If the target protein is not completely pure, a convenient alternative procedure is to purify the target protein by SDS-PAGE, transfer it to the PVDF blotting membrane supplied, cut out the band of interest, and perform the CNBr cleavage on the immobilized band (Figure 2).

The amount of target protein needed is determined by the type of sample available to the researcher and the size of the protein to be digested. If the target protein can be digested as a pure protein in solution, as little as 100pmoles can be used. This corresponds to 3µg of a 30,000 dalton protein or 10µg of a 100,000 dalton protein. If the sample protein is to be purified by SDS-PAGE and digested on an Immobilon-P membrane, 250pmoles or more of the target protein should be used to compensate for losses associated with the elution of peptides from the membrane. Table 8 shows the conversion of micrograms of target protein to picomoles of polypeptide.

Table 8. Conversion of Micrograms of Protein to Picomoles of Polypeptide.

Target Protein Molecular Weight	100pmoles (Solution Digestion Method)	250pmoles (SDS Gel Isolation Method)
100,000 daltons	10µg	25µg
50,000 daltons	5µg	12.5µg
10,000 daltons	1µg	2.5µg

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IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

Target proteins are cleaved to completion at the carboxylic sides of methionine residues by CNBr digestion in 70% formic acid. Fragments generated in this manner typically range in size from 12 to 250 amino acids, and are readily separated by the high resolution peptide separation gels used in this procedure. A very small amount of cleavage at Asp-Pro bonds may be seen as a result of the acidic conditions employed but, in general, this should not represent a difficulty since only a small percentage of cleavage should be seen at such sites. It is critical to the success of the system that the methionine residues be reduced; oxidized forms of methionine are refractile to cleavage with CNBr.

The steps required for preparation of samples for protein sequence analysis are illustrated here using the bovine serum albumin (BSA) positive control protein. To demonstrate the Probe-Design system procedure used when the protein sample requires SDS-PAGE purification, a 250pmole sample (16µg) of BSA was subjected to SDS-PAGE in four adjacent lanes (4µg each) and the protein bands were electroblotted to a PVDF blotting membrane. The transferred proteins were detected by Coomassie blue staining on the membrane. The BSA bands were excised, placed in 1.5ml microcentrifuge tubes and digested overnight at room temperature in a solution of CNBr in 70% formic acid. The resultant peptide fragments were then eluted from the membrane by extraction with a solution of isopropanol, water, trifluoroacetic acid, lysine and thioglycolic acid. The four eluted peptide samples were pooled, concentrated by drying and then resuspended in a small volume of SDS-PAGE loading buffer. The fragments were separated on a high resolution SDS-polyacrylamide peptide gel similar to that described by Shägger and von Jagow (6). The gel was stained with Coomassie blue to illustrate the separation of these fragments (Figure 3, lane 1). The apparent molecular weights of the CNBr-generated peptides were determined by comparison to Promega's low-range protein standards (lane 3), which are included with the system. When preparing peptides for protein sequencing, the gel-resolved fragments are directly electroblotted to a PVDF sequencing membrane and then stained with Coomassie blue. Finally, selected peptide bands are excised and submitted to a protein sequencing facility for analysis.

The CNBr fragments eluted from the Immobilon-P membrane need not be pooled into a single lane on the high resolution peptide separation gel. Lanes 4 and 5 show that somewhat better resolution was obtained when the CNBr fragments from 250pmoles of BSA were run in two lanes rather than one. For sequencing, the desired bands from two adjacent lanes can be excised from the ProBlott membrane and loaded side-by-side in the sequenator sample chamber.

Also illustrated in Figure 3 are the results of performing a CNBr digest on pure BSA in solution. In this case, 6.5µg (100pmoles) of BSA in 10mM Tris, pH 8.0 was placed in a 1.5ml microcentrifuge tube and digested with CNBr in formic acid. The solution was concentrated by drying and then dissolved in SDS-PAGE loading buffer. The redissolved peptides were fractionated by electrophoresis as described above (6), transferred

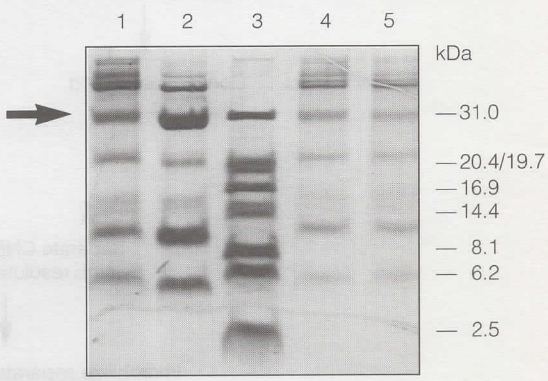

Figure 3. CNBr fragments of BSA resolved on a high resolution peptide separation gel. BSA was digested with CNBr either in solution or on a PVDF blotting membrane. The resulting fragments were concentrated, resuspended in sample buffer, resolved by electrophoresis on a high resolution peptide separation gel and stained with Coomassie blue. Lane 1, 250pmoles of BSA digested on a PVDF blotting membrane; lane 2, 100pmoles of BSA digested in solution; lane 3, 1.5µl of Promega low-range protein molecular weight markers, lanes 4 and 5, 250pmoles of BSA digested on PVDF blotting membrane and run in two adjacent lanes (125pmoles/lane).

IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

to a PVDF sequencing membrane, and detected by staining with Coomassie blue (Figure 3, lane 2). At this point, selected bands are ready to be excised with a razor blade, rinsed in deionized water, and submitted for protein sequence analysis. The membrane-immobiliized peptide bands may be stably stored dry at room temperature and may be placed in microcentrifuge tubes for mailing if a protein sequencing facility is not available locally.

Figure 3 shows that the same pattern of peptide fragments is obtained if CNBr digestion is performed in solution or on a PVDF blotting membrane. In the experiment shown, a slightly higher yield of peptides was obtained from the CNBr digest in solution, despite the greater amount of starting material used for the digest on the solid support. In both cases, however, the amount of material in the stained peptide was ample for protein sequence analysis (10-40pmoles of released amino acid in the first sequenator cycle). The recovery of peptide fragments after digestion on the PVDF solid support appears to be dependent on the protein that is digested; recovery of fragments from many proteins may be better than that observed with BSA, as BSA is known to be difficult to elute from membranes.

Relative Advantages of the Two Types of PVDF Membrane Supplied

The two types of PVDF membranes included with the Probe-Design system offer different advantages for the generation and separation of peptide fragments. Our studies indicate that PVDF blotting membrane gives better recovery of eluted peptides after digestion of target proteins on a solid support. In our experience, the PVDF sequencing membrane supplied is superior for electroblotting of small peptides, resulting in significantly higher levels of signal when used as the solid support for protein sequence analysis. Gloves should be worn whenever either of the membranes are being handled to avoid contamination of the sequencing samples with other polypeptides.

Prevention of Problems Due to N-Terminal Blockage

Since such small amounts of protein are being digested when this kit is used, very small amounts of reagents that can react with free amino groups can chemically block the new amino termini of the peptides generated during CNBr digestion. If this occurs, a set of unsequencable peptides may be generated from your target protein. This problem can be particularly serious when gel fractionation methods are used. To minimize chemical blockage of peptides, the Probe-Design system protocol employs protective agents during the elution, fractionation and electroblotting steps to help ensure that peptides are not chemically blocked prior to protein sequence analysis. Lysine, a source of free amino groups, is added to the elution step to help prevent chemical blocking of the new N-termini generated during the CNBr cleavage reaction. To prevent blockage of the amino termini of peptides during SDS-PAGE, the gel is left at room temperature for at least two hours following polymerization. This allows amino-reactive groups generated during polymerization to react with the gel components, thus preventing them from reacting with the amino termini of the peptides. In addition, thioglycolic acid, a free radical scavenger, is added to the cathode buffer and pre-electrophoresed into the gel before the peptides are applied. The glycine present during the electroblotting steps also serves as a source of protective free amino groups. One consequence of the presence of glycine, however, is that residual glycine in the PVDF sequencing membrane may appear in the first cycle of sequencing (Figure 4). Other transfer buffers, such 10mM CAPS, pH 11, have been used for electroblotting, but these do not provide protection of N-termini. While the Probe-Design system procedure protects new N-termini, it will not unblock the original amino terminus of a protein that has been chemically blocked before cleavage of the protein. This should not be a difficulty in using the system, as several different fragments are usually obtained. Thus if one fragment is blocked, other fragments can provide the necessary sequence information.

IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

B. Applications of Protein Sequence Analysis

Design of Oligonucleotide Probes from Protein Sequence Data

The partial protein sequence of the target protein can be used to design oligonucleotide probes by reverse translation of the protein sequence. It is usually best to have more than one protein segment sequenced in order to obtain a protein sequence that will have the lowest possible redundancy in codon usage.

The sequences of such probes are typically designed by deducing the nucleotide sequence that would be needed to encode the peptide using the genetic code appropriate for the system under study. In general, probes 15-20 bases in length are used for screening cDNA or genomic libraries (9). Thus, unambiguous stretches of 5-6 amino acids must be obtained by protein sequencing. Due to the redundancy of the genetic code, it is usually impossible to obtain a peptide sequence that would be encoded by just one nucleotide sequence. Probes designed from protein sequence data are generally synthesized to contain a mix of all the possible coding sequences that would be capable of encoding the target peptide. In these cases, the probe must be designed using the stretch of 5-6 amino acids with the fewest possible coding sequences. Table 9, below, indicates the number of codons that encode each commonly found amino acid. For a complete codon usage table, refer to pg. 393.

Table 9. Number of Codons that Encode the Commonly Found Amino Acids as Determined Using the Universal Genetic Code.

Number of Codons	Amino Acids
1	Met, Trp
2	Phe, Tyr, His, Gln, Asn, Lys, Asp, Glu, Cys
3	Ile
4	Val, Pro, Thr, Ala, Gly
6	Leu, Ser, Arg

Figure 4, (pg. 261) illustrates the design of oligonucleotide probes from protein sequence data obtained by sequencing the 28kDa peptide fragment of BSA indicated by the arrow in Figure 2. The published sequence of this portion of BSA contains a methionine at the N-terminal end of the fragment sequence obtained, verifying that this 28kDa fragment was generated by CNBr cleavage. The two candidate probe sequences shown in Figure 4 were chosen on the basis of minimizing the redundancy in codon usage while maximizing the length of the probe designed. Obviously, the amino acids that have six possible codons are extremely undesirable for designing a probe sequence. In cases where one of these amino acids must be used to design the probe sequence (Figure 4, cycle 5), it is helpful to order two probe sequences. For example, the codons for Leu in the universal genetic code are: TTA, TTG, CTA, CTG, CTT and CTC. If only one probe for a peptide encoding a Leu were ordered, the probe would have to be designed with the following ambiguities:

```
      A
C T G
T   C
    T
```

However, this mixture of probe sequences would also encode Phe codons (TTT and TTC) at the position of the Leu residue. In such cases, therefore, it is better to order two probe sequences that differ in the nucleotides encoding the Leu residue. One probe sequence would have the Leu position encoded by the CTN codons and the other would contain the TT(A/G) codons for Leu.

IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

In most cases, the amino acid residue preceding the derived sequence will be a methionine since CNBr was used to fragment the protein. If protein sequencing does not yield a low redundancy probe, one may consider incorporating the codon for methionine at the proper position in the probe sequence. However, it is essential to realize that this assumption can be incorrect if the fragment being sequenced is the N-terminal fragment of the target protein or if cleavage of the protein has occurred at an unexpected location. This is particularly important to remember if the derived peptide sequence begins with a Pro residue, for small amounts of acid cleavage at Asp-Pro sequences may occur during the digestion of the protein with CNBr.

Other methods are available for increasing the specificity of probe sequences. These take advantage of the codon bias of specific organisms or the nucleotide ratio (G+C vs. A+T) of the host species. A very good review of these approaches is found in Sambrook, *et al.* (18).

Since probes designed from protein sequence information will virtually always be degenerate, they will commonly hybridize with multiple clones in a cDNA or genomic library. When this occurs, probes designed from different stretches of the target protein sequence can be used to perform confirmatory screening of clones. Although some protein segments may prove to be poor candidates for designing probe sequences, the information obtained in determining their sequence is not wasted. These peptide sequences can be used to verify that the correct clone has been obtained; they should be encoded in the sequence of the cloned DNA fragment.

Oligonucleotide Probe Design

Sequence Cycle		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sequence Obtained		Gly Arg	Glu	Lys	Val	Leu	Thr	Ser	Ser	Ala	Arg	Gln	Arg	Leu	Arg
Probe Designed			GAA G	AAA G	GTN	TTA G CTN	ACN								
Published Sequence	Met	Arg	Glu	Lys	Val	Leu	Thr	Ser	Ser	Ala	Arg	Gln	Arg	Leu	Arg

Sequence Cycle	15	16	17	18	19	20	21	22	23
Sequence Obtained	-	Ala	Ser	Ile	Gln	Lys	Phe	Gly	Glu
Probe Designed				ATA C T	CAA G	AAA G	TTT C	GGN	GAA G
Published Sequence	Cys	Ala	Ser	Ile	Gln	Lys	Phe	Gly	Glu

Figure 4. Illustration of oligonucleotide probe design using protein sequence information obtained using the Probe-Design peptide separation system. A portion of the amino acid sequence for the 28kDa BSA fragment indicated in Figure 3 (arrow) is shown along with two possible probe sequences designed using this information. The published sequence for this region of the protein is also shown. Note that the first cycle contained significant amounts of both arginine and glycine. The presence of glycine, however, can be attributed to residual glycine from the electroblotting step. No amino acid was called at cycle 15. The letter "N" in the probe sequence indicates that a mixture of all four nucleotides would be included in the probe at that site.

Protein Analysis

IV. Preparation of Proteins for Sequencing using the Probe-Design™ Peptide Separation System

(continued)

Additional Applications for Protein Structural Analysis

The Probe-Design peptide separation system is also useful for localizing sites of protein modifications. For example, protein phosphorylation sites labeled with ^{32}P may be localized to a specific CNBr peptide fragment by autoradiography. The sequence of that PVDF-immobilized peptide can then be determined. Since most phosphorylation sites in proteins are stable in acid solutions, ^{32}P typically remains associated with the peptide being sequenced. In some cases, the exact location of a phosphorylated site has been determined by following the release of radioactivity during sequential Edman degradations in a protein sequenator (19,20), but this has required modifications of standard sequencing procedures. It is also possible to determine the exact location of a radiolabeled amino acid (such as iodoacetamide-labeled cysteine) during protein sequencing. The derivatized amino acid must be uncharged, however, or it may bind to the filter supporting the PVDF membrane (21).

In a related application, a specific epitope may be localized to a specific CNBr fragment immobilized on the ProBlott membrane. A monoclonal antibody-based Western blot is used on a portion of the blot to detect the band of interest. That band is then excised from a lane run in parallel and protein sequence information is obtained (22). For this method to be effective, the monoclonal antibody used must be able to recognize primary structure antigenic determinants and the epitope to be recognized can not contain a methionine residue.

One limitation to localizing protein modifications with the Probe-Design system is that, in many cases, the entire sequence of a given fragment will not be obtained. Using liquid-digested samples, we have been able to sequence fragments purified by this system for 20 to 30 cycles. However, cyanogen bromide fragments average around 50 to 60 amino acids in length. Thus if a modification is present beyond the length of sequence that can be obtained, it could not be located precisely by this method.

V. Western Blot Detection of Proteins with the ProtoBlot® AP and HRP Systems

A. General Considerations

The ProtoBlot® Western Blot AP and HRP systems are designed for the rapid, sensitive detection of proteins or other macromolecular antigens immobilized on nitrocellulose membranes. These antigens may either be transferred from gels after electrophoresis (Western blots; 23,24) or bound directly from solution ("dot" blots). The method is based on the enzyme-linked immunodetection of antigen-specific antibodies (supplied by the researcher) using anti-IgG second antibodies conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP). Systems are available for the detection of human, mouse, rat and rabbit antibodies. Following sequential incubations with the primary antibody and appropriate anti-IgG enzyme conjugate, a solution containing the color development substrate(s) is added. Sites of antigen localization turn a dark purple color as the result of the localized enzyme activity. In most cases, blotted proteins can be visualized in 2-3 hours with this method.

While other detection systems such as those based on ^{125}I-protein A have been used successfully, the use of enzyme conjugates offers significant advantages in convenience and safety. Promega enzyme conjugates are affinity-purified on immobilized IgG and concentrated to very high titers to yield maximum sensitivity of detection with extremely low background.

For many applications, AP conjugates are superior to HRP conjugates in that they offer greater sensitivity of detection, are not inhibited by azide, and employ a substrate that is not prone to fading during long-term storage (25,26). HRP conjugates are a sensitive and proven alternative to AP and should be used when endogenous phosphatase activity or phosphate ions could interfere with interpretation of the blot.

Antigens can be immobilized on nitrocellulose membranes ("blotted") by one of several methods, depending on the requirements of the experiment. In all cases, the ability to detect a given antigen will depend on the amount of antigen per unit area of the membrane and on the characteristics of the primary antibody. As a guideline, it has been possible to reliably detect 10-50pg of antigen (using AP-conjugated second antibodies) or 0.5-1.0ng of antigen (using HRP-conjugated second antibodies) spotted in 1μl (about $4mm^2$) using this system and reasonably high affinity, high titer primary antibodies.

Several considerations should be taken into account when developing a blotting assay for a given antigen:antibody combination. For example, some antibodies (particularly monoclonals) recognize epitopes that may become buried or denatured when the antigen is bound to surfaces such as nitrocellulose. This effect is exaggerated

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(continued)

when blotting protein antigens out of SDS containing gels. The sensitivity of the assay with even a "good" antibody can often be several fold lower on Western blots (using denaturing gels) than on "dot" blots where native proteins are spotted directly on the membrane.

In practice, it is useful to blot a series of known amounts of antigen by the method required for the experiment and test several dilutions of primary antibody to maximize sensitivity and minimize background. If the primary antibody is used at too high a concentration, high backgrounds can result. If the primary antibody concentration is too low, the positive signals will be weaker. In general, the optimal dilutions for most sera, ascites fluids, and purified antibodies will be in the range of 1:200 to 1:10,000. Hybridoma tissue culture fluids (which have much lower antibody concentrations) usually require dilutions from 1:10 to 1:100 for optimal performance.

B. Detection of Blotted Antigens

Reagents to be Supplied by the User

Instructions for preparing these reagents are provided on pg. 268.

- TBS buffer
- TBST buffer
- AP buffer
- AP color development solution
- HRP color development solution

1. Bind the antigen to the nitrocellulose membrane. Three different methods are described below.

   Dot Blotting

   A solution containing the antigen (crude or purified) is spotted directly on a dry or moist membrane, usually in a 1µl volume. To prepare a moist membrane, float the membrane in a container of TBS buffer (without Tween® 20*) until it is evenly wet, submerge it briefly, and place it on a piece of dry filter paper. Let the excess buffer drain for about 5 minutes before use. Dry membranes can be placed on top of filter paper or the backing paper included with the nitrocellulose membranes. It is useful to mark the membrane with a pencil or ball point pen at convenient intervals as a guide in spotting. Prepare appropriate dilutions of the sample in TBS and apply in 1µl volumes to the membrane using a micropipet. If more volume is required, the sample can be applied in multiple applications over the same spot. Each spot should be allowed to dry thoroughly before the next volume is applied. Allow all of the spots to dry before proceeding to the blocking step. An alternative method for making dot blots is to use a filtration manifold which allows multiple samples to be applied simultaneously in uniform spots. Several devices are commercially available for this purpose.

*Tween is a registered trademark of ICI, Inc.

Transfer from Gels, Western Blotting

Antigens are transferred from polyacrylamide or agarose gels to the membrane by passive diffusion or electrophoresis. Western blots are usually made by electrophoretic transfer of proteins from SDS-polyacrylamide gels. Detailed procedures for electrophoretic blotting are usually included with commercial devices and also can be found in references 23, 27 and 28.

Plaque/Colony Lift Immunoscreening

A detailed procedure for screening lambda vector expression libraries with antibodies is described on pg. 221. Procedures for making colony lifts when immunoscreening plasmid libraries have been described by Esen, *et al.* (29) and Helfman, *et al.* (30).

Notes:

a. At this point, the membranes can be stored, wrapped in plastic, at 4°C.

b. Do not allow the membranes to dry out during any of the subsequent steps.

c. Perform all of the subsequent washing and incubation steps at room temperature with gentle shaking. It is convenient to use a shallow container that is slightly larger than the membrane.

d. For antibody incubations and the color development reaction, use just enough solution to submerge the membrane, protein side up. Usually, this volume is about 0.1-0.15ml/cm^2 of membrane surface (25ml for a 15x15cm membrane). Use at least twice this volume for blocking and washing steps.

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2. Float the membrane on TBST (pg. 268) until it is evenly wet, submerge it, and rinse briefly in the same buffer. To saturate nonspecific protein binding sites, decant the buffer and incubate the membrane in blocking solution for 30 minutes with agitation.

3. Replace the blocking solution (which can be reused several times) with TBST containing the desired dilution of primary antibody and incubate for 30 minutes with gentle agitation.

4. To remove unbound antibody, wash the membrane in TBST three times for 5-10 minutes each.

5. Transfer the membrane to TBST containing the appropriate anti-IgG enzyme conjugate and incubate for 30 minutes with gentle agitation. We recommend using a 1:7,500 dilution of the anti-IgG-AP conjugate or a 1:2,500 dilution of the anti-IgG-HRP conjugate.

6. Wash the membrane in TBST three times for 5-10 minutes each.

7. Perform the AP or HRP color reaction as follows:

AP Color Reaction

Prepare 10ml of AP color development solution. Blot the damp membrane dry on filter paper and then transfer it to the AP color development solution. Reactive areas will turn purple, usually within 1-15 minutes. Color development will continue for at least 4 hours. Membranes can be developed overnight, but may have high backgrounds as a result.

HRP Color Reaction

Prepare 10ml of HRP color development solution. Blot the membrane damp dry on filter paper and transfer it to the HRP color development solution. Reactive areas will turn purple. Color development is generally complete within 30 minutes.

8. When the color has developed to the desired intensity, stop the reaction by rinsing the membrane in deionized water for several minutes, changing the water at least once. The membrane can be photographed while still moist by placing it on top of a damp piece of filter paper on a glass plate. For storage, the membrane can be air dried on filter paper. The AP substrate color will fade slightly upon drying but can be restored by moistening with water. The HRP substrate color will fade with time but can be partially restored by moistening. Protect the membrane from light during prolonged storage.

V. Western Blot Detection of Proteins with the ProtoBlot® AP and HRP Systems

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C. Troubleshooting

Symptoms	Possible Causes	Comments
Signal weak or absent.	Antibody lost activity during storage or is of low titer.	Store antisera/ascites at −20°C in aliquots and avoid repeated freeze/thaw cycles. Use some other immunochemical assay, such as ELISA, immunoprecipitation, immunodiffusion, etc., to confirm that the antibody has reactivity toward the antigen. If this is positive, repeat the blot assay using a higher concentration of primary antibody.
	Antibody is of low affinity.	Low affinity antibodies are more affected by buffer conditions, incubation times, and relative concentrations than are high affinity antibodies. Eliminate Tween 20 from the buffers and increase the incubation time and the concentration of the primary antibody.
	Antibody activity is conformation-dependent.	Some antibodies (particularly monoclonals) recognize epitopes involving secondary and/or tertiary structures that are altered when the antigen is applied to nitrocellulose (see pg. 262, Section V.A). Test the antibody with dot blots made with native and denatured antigen. In some cases, native antigens produce a positive signal, whereas SDS-denatured antigens are completely negative. In other cases, an antibody that recognizes an antigen in solution (i.e., will immunoprecipitate the antigen) will not recognize the same antigen when it is bound to a surface, even under nondenaturing conditions. These antibodies are generally negative in ELISA as well as in blotting assays.
	Inefficient transfer of antigen out of gel.	Stain the gel with Coomassie blue or silver stain after transfer to monitor the disappearance of antigen from the gel. If too much antigen remains in the gel, longer transfer times or higher voltages may be required.
	Inefficient binding of antigen to membrane.	This can be caused by insufficient contact or air bubbles between the gel and the membrane, or other conditions of transfer such as pH (more important to consider with native gels) or excess amounts of SDS in the gel or buffer. Proteins may be stained directly on nitrocellulose membranes with 0.2% Ponceau S, India ink (31) or with 0.1% Amido black in 20% methanol, 10% acetic acid. Alternatively, an extremely sensitive staining method involves derivatization of bound proteins with 1-fluoro-2, 4-dinitrobenzene followed by

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Symptoms	Possible Causes	Comments
Signal weak or absent. (continued)		incubation with rabbit anti-dinitrophenol antiserum, AP- or HRP-conjugated goat anti-rabbit IgG, and color development with NBT/BCIP or 4-chloro-1-naphthol (an adaptation of the method described in references 32 and 33, pg. 269).
	Anti-IgG enzyme conjugate activity is too low.	Conjugate improperly stored. Store at 4°C or in aliquots at −20°C. Avoid repeated freeze/thaw cycles, heat treatment and bacterial contamination. Test activity by adding 1ml of the recommended dilution of enzyme conjugate (Section B, pg. 264) in TBST to 1ml of color development solution. Intense purple color should appear within 5 minutes. (This is also a test for activity of the color development substrates.) Although unlikely, it is possible that some water may contain inhibitors. Use reagents of the highest quality available.
	Improper blocking agent was used.	Some blocking proteins may have effects on antigen recognition by certain antibodies. If this is suspected, try a blocking protein other than BSA (such as casein, gelatin, or serum) to saturate excess binding sites. If an agent other than ProtoBlot BSA is used, include controls omitting the primary antibody, the anti-IgG conjugate and both antibodies from the procedure to check for possible effects on background.
General purple background throughout the membrane.	Color development reaction was too long.	Stop the reaction when the color has reached the desired intensity.
	Poor quality nitrocellulose.	Use a different source of nitrocellulose.
	Poor quality anti-IgG conjugates.	Use affinity-purified ProtoBlot western blotting system AP or HRP second antibodies.
	Improper blocking.	Use ProtoBlot BSA. The blocking step incubation time can be increased to 1-2 hours if necessary. Some alternative blocking agents such as non-fat dry milk may contain IgG or phosphatase or peroxidase activity which could bind to the enzyme conjugates. Perform controls by omitting the primary antibody and the anti-IgG conjugate from the procedure.
	Anti-IgG enzyme conjugate concentration too high.	Use the second antibody at the recommended dilution. Generally, the background will increase significantly at concentrations higher than 1:2,500 for the AP conjugate or 1:1,000 for the HRP conjugate.

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Symptoms	Possible Causes	Comments
General purple background throughout the membrane. (continued)	Primary antibody sticks nonspecifically even to blocked membranes.	Perform the procedure using no antigen (just a blocked membrane). A few IgG and other Ig (particularly IgM) molecules that will be recognized by the anti-mouse and anti-human AP conjugates are especially "sticky" and may be difficult to use for blotting.
Localized "background": unexpected spots or bands appear to be positive.	Sample has phosphatase or peroxidase activity.	Omit the primary antibody and anti-IgG AP or HRP conjugate incubations from the procedure. If color development is significant, try to inactivate the enzyme activity in the sample by either heating the blot at 80°C for 20 minutes, or incubating it in 0.1M acetic acid for 20 minutes followed by rinsing in TBST prior to the blocking step. If this treatment is successful, check that the treatment does not inhibit primary antibody binding.
	Primary antibody recognizes epitopes shared by other species in the sample or contains a mixture of antibodies with multiple specificities.	If the sample contains a mixture of antigens, the primary antibody may either contain IgG that recognizes molecules other than those being assayed, or the same IgG may cross-react with other molecules due to shared epitopes. It is sometimes difficult to distinguish between these possibilities, but several approaches are possible (for a more detailed discussion, see reference 29, pg. 269). The most straightforward is to design preadsorption experiments in which the primary antibody is incubated with various samples prior to being used in a blotting assay. For example, preincubate a crude sample not containing the antigen of interest with the antibody at various ratios, and then perform a blotting assay using the original crude antigen as the sample. If the antibody was heterogeneous and contained IgG directed against other antigens, the preadsorption (at high ratios) should remove those specificities without affecting the IgG of interest. As a result, samples on the blot should be positive and the background should be decreased relative to unadsorbed antibody. However, if the antibody recognizes epitopes shared by other antigens, the preadsorption (at some ratio) should remove the IgG of interest since it is the same IgG that reacts with the antigen being assayed. In this case, the signal using the preadsorbed antibody will be decreased. In a reciprocal experiment, highly purified antigen could be used to preadsorb the antibody, and the results of the blotting assay should complement those using the crude mixture for adsorption.

V. Western Blot Detection of Proteins with the ProtoBlot® AP and HRP Systems

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Composition of Solutions

TBS (Tris-buffered saline):

10mM Tris-HCl, pH 8.0
150mM NaCl

TBST buffer:

TBS + 0.05% Tween 20

Blocking solution:

TBST + 1% (w/v) BSA

For horseradish peroxidase only:

HRP color development solution:

2ml methanol (reagent grade)
50µl 4-chloro-1-naphthol (100mg/ml stock in methanol)
8ml TBS
35µl H_2O_2 (30% solution)

Add 4-chloro-1-naphthol solution to 2ml methanol, then add TBS and mix. Add H_2O_2 and mix again.

For alkaline phosphatase only:

AP buffer:

100mM Tris-HCl, pH 9.5
100mM NaCl
5mM $MgCl_2$

AP color development solution:

10ml AP buffer
66µl NBT (nitroblue tetrazolium) stock solution
33µl BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock solution

Add NBT solution to AP buffer, mix. Then add BCIP solution and mix again. Protect the solution from strong light and use within 1 hour of preparation. If either of the substrates has precipitated during storage, warm the substrate vial to room temperature and mix to redissolve before use.

VI. References

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VII. Additional Protein Analysis Literature Available from Promega

Technical Bulletins

089	Protein Fingerprinting System
100	ChromaPhor™ Protein Visualization System
2003	Alkaline Protease
2005	Endoproteinase Glu-C
2006	Endoproteinase Lys-C
2012	Modified Trypsin
2013	Proteinase K

Manuals

ProtoBlot® Western Blot AP System Technical Manual

ProtoBlot® Western Blot HRP System Technical Manual

RapidChanger™ Size Exclusion Cartridges Technical Manual

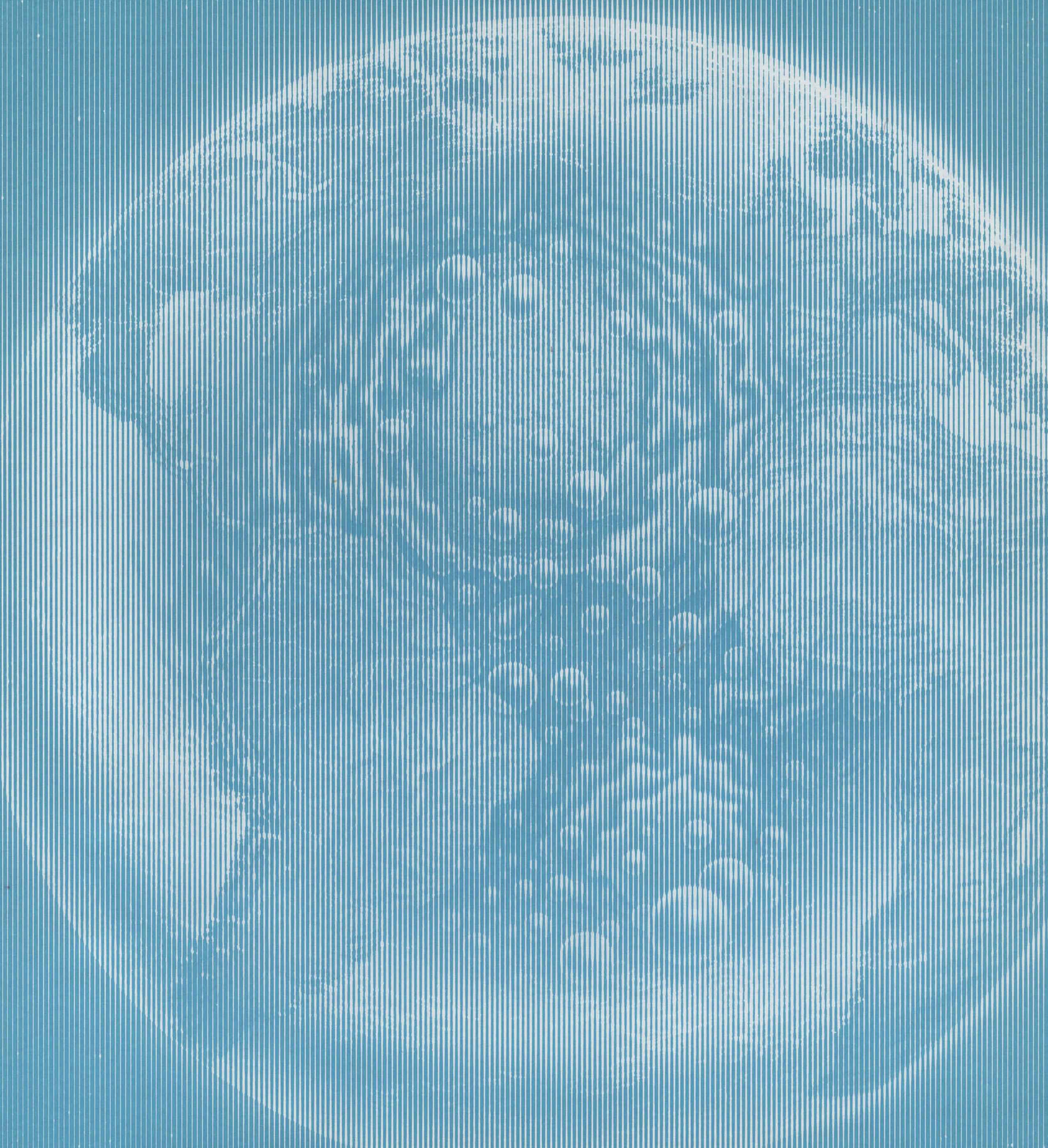
Probe-Design™ Peptide Separation System Technical Manual

Promega Notes Articles

Issue	Title
3	Immunodetection using alkaline phosphatase conjugated second antibodies
4	ProtoBlot® immunoblotting system
5	Monoclonal antibody binding to whole mounts of hydra using alkaline phosphatase-conjugated second antibody
24	Protein fingerprinting
26	Design of oligonucleotide probes: preparation of proteins for sequencing with the Probe-Design™ peptide separation system
29	ChromaPhor™ protein visualization system

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Cellular Regulation

Contents

I. Properties and Applications of Growth Factors and Cytokines

The growth and differentiation of mammalian cells are regulated by a host of extracellular signals, including growth factors and cytokines. Growth factors and cytokines are soluble cell products that can act locally to influence the growth and behavior of target cell populations.

Cytokines

The scientific literature contains conflicting definitions of cytokines and lymphokines. Cytokines are commonly defined as a class of soluble protein factors which are secreted by cells of hematopoietic lineage and which trigger a variety of physiological responses, in particular immune responses (1). Lymphokines are defined as a subset of cytokines which are produced specifically by lymphocytes. Unlike immunoglobulins, lymphokines do not exhibit specificity for antigens. Interestingly, many lymphokines are also produced by cells other than lymphocytes. The term "interleukin" was coined for those factors that mediate communication between leukocytes, but the more general term lymphokine is commonly applied to these factors (2). Examples of cytokines that are not lymphokines are the tumor necrosis factors and colony stimulating factors. Most cytokines isolated from their natural sources are glycosylated, but recombinant, unglycosylated forms are also active on target cells. Several properties of the better-studied cytokines and lymphokines are listed in Table 1, pg. 272.

The Biological Response Modifiers Program (BRMP) of the National Institutes of Health and the National Institute of Biological Standards and Controls (NIBSC) of the World Health Organization have assigned standard BRMP units of bioactivity to many human cytokines. As these units are assigned, the specific activities of Promega's human cytokines will be expressed as BRMP units. Where possible, we will use the same cell lines and assay protocols used by those organizations.

Growth Factors

Growth factors are soluble polypeptides (5,000-35,000MW) produced by a variety of cells. While their common names reflect the activity or isolation source originally described, growth factors have come to be recognized as multifunctional. A given growth factor may stimulate proliferation of certain cell types, inhibit proliferation of others, and cause effects unrelated to proliferation in additional cell types (3). Growth factors act by binding to specific cell surface receptors and triggering a cascade of intracellular events necessary for proliferation or other effects (4). Many growth factor receptors contain tyrosine kinase activity which, upon growth factor binding, phosphorylates the receptor and/or other intracellular target proteins (5,6). The identification of the phosphorylated proteins can be an important first step in studying the mechanism of signal transduction.

In addition to their usefulness for studies of cellular growth and regulation, growth factors are important as growth-enhancing supplements in defined, serum-free culture media. In many cases, the analogous growth factors isolated from different species have conserved receptor-binding regions and exhibit cross-species activity.

Growth factor mechanisms of action are closely tied to oncogenesis. Several oncogene products lead to increased cell responsiveness to growth factors or are themselves altered forms of growth factors or growth factor receptors, resulting in unregulated cell division (7,8). Several properties of the better-studied growth factors are listed in Table 2, pg. 274.

Cellular Regulation

I. Properties and Applications of Growth Factors and Cytokines

(continued)

Table 1. Properties of Cytokines. The molecular structure, target cells and biological effects of several cytokines are listed below. The properies given are for human factors, unless specified otherwise. Factors listed in boldface type are available from Promega.

Name	Molecular Structure	Other Names	Target Cells	Biological Effects
G-CSF	18-32kDa glycoprotein	DF, MGI-2, GSF-β, pluripoietin	Granulocyte Progenitors	Maturation of granulocyte progenitors.
M-CSF	70-90kDa glycosylated dimer	CSF-1	Macrophage progenitors	Maturation of macrophage progenitors.
GM-CSF				
human, recombinant	14kDa, 123aa (Natural form is an 18-22kDa glycoprotein.)	CSF-2, neutrophil migration inhibitory factor, pluripoietin-α	Mature and progenitor granulocytes and macrophages, eosinophils, neutrophils	Maturation of granulocyte and macrophage progenitors; proliferation of mature granulocytes, macrophages, and eosinophils. No human-mouse cross-species activity.
mouse, recombinant	13.5kDa, 124aa (Natural form is a 22-23kDa glycoprotein.)			
TNF-α human, recombinant	17kDa subunit, 157aa (occurring as a dimer, trimer, or pentamer)	Cachectin	Lymphoma cells, polymorphonuclear leukocytes, fibroblasts, epithelial cells, endothelial cells	Cytolysis or cytostasis of tumor cells, activation of polymorphonuclear leukocytes, antiviral activity, induction of IL-1 or colony stimulating factor expression.
TNF-β human, recombinant	19kDa, 171aa	Lymphotoxin	Lymphoma cells, polymorphonuclear leukocytes, fibroblasts, epithelial cells	Cytolysis or cytostasis of tumor cells, activation of polymorphonuclear leukocytes, antiviral activity.
MCAF human, recombinant	8.5kDa, 76aa	MCP-1	Tissue macrophages, monocytes	Inflammatory response of blood monocytes and tissue macrophages.
IL-1α human, recombinant	17kDa, 159aa (Natural form is an 18kDa glycoprotein.)	Lymphocyte activating factor, T cell replacing factor, B cell activating factor, B cell differentiation factor	T cells, B cells, macrophages, neutrophils, fibroblasts, synovial cells, hepatocytes	Multiple effects, including lymphokine release by T cells; B cell differentiation; augmentation of cytotoxicity; prostaglandin and collagenase release from macrophages, fibroblasts, and synovial cells. Exhibits human-mouse cross-species activity, though with reduced intensity of response.
IL-1β human, recombinant	17kDa, 153aa (Natural form is a 17kDa glycoprotein.)	See IL-1α.	See IL-1α.	See IL-1α.
IL-2 human, recombinant	15kDa, 133 aa (Natural form is a 15-30kDa glycoprotein.)	T cell growth factor, thymocyte stimulation factor, thymocyte mitogenesis factor, T cell replacing factor, killer helper factor	T cells, B cells, cytotoxic NK cells, lymphokine activated killer cells	Proliferation and activation of T cells, B cells, NK cells, and lymphokine activated killer cells.

Promega

(continued on next page)

I. Properties and Applications of Growth Factors and Cytokines

(continued)

Name	Molecular Structure	Other Names	Target Cells	Biological Effects
IL-3 human, recombinant	15kDa, 133aa (Natural form is a 20kDa glycoprotein.)	Multi-CSF, CSF-2α, CFU-3A, mast cell/ leukocyte growth factor, cell stimulating factor, burst promoting activity	Granulocyte, macrophage, neutrophil and erythrocyte progenitors; megakaryocytes; mast cells; T cells; B cells	Differentiation and proliferation of macrophages, neutrophils, and megakaryocytes from multipotential progenitors; T cell growth; IgG secretion. No mouse-human cross-species activity.
IL-4	15-20kDa glycoprotein	B cell growth factor, B cell stimulatory factor-1, T cell growth factor-2, mast cell growth factor	T cells, B cells, macrophages, mast cells, myeloid progenitors	B cell expression of MHC Class II molecules and immunoglobulins, growth of T cells and mast cells. No mouse-human cross-species activity.
IL-5	45kDa glycosylated dimer	T cell replacing factor, eosinophil differentiating factor, B cell growth factor II	B cells, eosinophils, thymocytes	Production of eosinophils; differentiation and proliferation of B cells; generation of cytotoxic T cells.
IL-6 human, recombinant	20.5kDa, 184aa (Natural form is a 23-30kDa glycoprotein.)	IFN-β2, BSF-2, HSF, PCT-GF, IL-HP1, hybridoma growth factor	T cells, B cells, myeloma cells, thymocytes, hematopoietic stem cells, hepatocytes	Proliferation of hybridoma and plasmacytoma cells, thymocytes and granulocyte/macrophage progenitors; differentiation of B cells; generation of cytotoxic T cells.
IL-7 human, recombinant	17kDa, 152aa (Natural form is a 15-25kDa glycoprotein.)	Lymphopoietin-I	B cell and T cell progenitors	IL-2 production and T cell receptor expression, proliferation but not maturation of B cell and T cell progenitors.
IL-8				
human, recombinant (endothelial)	8.5kDa, 77aa	Neutrophil-activating peptide, monocyte-derived neutrophil chemotactic factor	Neutrophils, lymphocytes	Chemotaxis of neutrophils and lymphocytes.
human, recombinant (monocyte)	8kDa, 72aa			
Erythropoietin	34-39kDa glycoprotein	EPO	Red blood cell progenitors	Differentiation of red blood cell progenitors to erythroblasts.
IFN-α	17-21kDa dimer	Leukocyte IFN, type I IFN, viral IFN	NK cells, lymphocytes	Inhibition of viral infection, stimulation of MHC Class I antigen expression.
IFN-β	15-25kDa	Fibroblast IFN, type I IFN, viral IFN	Lymphocytes	Inhibition of viral infection.
IFN-γ human, recombinant	17kDa, 143aa (Natural form is a dimer of two 20-25kDa glycoproteins.)	Immune IFN, type II IFN	Monocytes, lymphoid cells, mast cells, endothelial cells, fibroblasts, neuronal cells, melanocytes, macrophages, NK cells, myelomonocytic cells, B cells	Inhibition of viral infection, induction of MHC Class I and II antigen expression, activation of NK cells and LAK cells.

Cellular Regulation

I. Properties and Applications of Growth Factors and Cytokines

(continued)

Table 2. Properties of Peptide Growth Factors. Peptide growth factors stimulate or inhibit growth of target cells by binding to specific cell surface receptors. Many growth factor receptors contain an intracellular tyrosine kinase activity which is stimulated by growth factor binding to the extracellular moiety of the receptor. The activated kinase phosphorylates the receptor as well as other intracellular protein targets. For other growth factors, the mechanism of signal transduction is unknown. Factors listed in boldface type are available from Promega.

Name	Molecular Structure	Other Names	Target Cells	Biological Effects
EGF				
human, recombinant	6kDa, 53aa	Urogastrone	Endoderm and ectoderm derived cells such as mammary, cornea, epidermis, liver, pancreas, nerve, glial, amniotic and adrenal medulla cells	Activation of EGF receptor tyrosine kinase and protein kinase C, modulation of proliferation in a variety of cell types.
mouse, natural	6kDa	—		
acidic FGF				
human, recombinant	15.5kDa, 140aa	HBGF-1, ECGF	Most mesoderm, neuroectoderm and some epithelial derived cells	Activation of FGF receptor tyrosine kinase, proliferation of target cells, angiogenesis.
bovine, natural	mixture of 16kDa and 18kDa polypeptides		Vascular endothelial cells, mouse 3T3 fibroblasts, human prostate cells	
basic FGF human, recombinant	17kDa, 154aa	HBGF-2	Vascular and corneal endothelial cells, mouse 3T3 fibroblasts, human prostate cells, mammary cells, chondrocytes, osteoblasts, myoblasts, smooth muscle and glial cells	Activation of FGF receptor tyrosine kinase, proliferation of target cells in a variety of tissues, angiogenesis.
IGF-I human, recombinant	7.5kDa, 70aa	Somatomedin-C	Mammalian and avian fibroblasts, GH3 cells, chondrocytes, adipocytes, sertoli cells, granulosa cells and other cells of mesenchymal origin	Activation of type I IGF receptor tyrosine kinase, sulfation of cartilage, insulin-like activity, proliferation of fibroblasts.
IGF-II human, recombinant	7.5kDa, 67aa	MSA	See IGF-I.	Exerts effects through type II IGF receptor.
2.5S NGF mouse, natural	14kDa, β subunit	—	Sympathetic and sensory neurons, normal and transformed chromaffin cells, PC-12 cells	Maintenance of nerve cells in culture, neurite extension. Unknown signal transduction pathway.
7S NGF mouse, natural	130kDa, $\alpha_2\beta\gamma_2$ subunit mixture	—	See 2.5S NGF.	See 2.5S NGF.

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I. Properties and Applications of Growth Factors and Cytokines

(continued)

Name	Molecular Structure	Other Names	Target Cells	Biological Effects
PDGF(AB) human, natural	30kDa, glycosylated AB heterodimer	—	Mesoderm derived mammalian and avian cells, brain glial cells, dermal fibroblast, arterial smooth muscle cells, some mammalian adenocarcinomas	Activation of PDGF receptor tyrosine kinase, phosphatidyl inositol turnover, proliferation of mesenchymal and smooth muscle cells.
PDGF(AA) human, recombinant	26.5kDa AA homodimer, 250aa 14-18kDa subunit		Mammalian and avian cells of mesodermal and neuroectodermal origin such as fibroblasts, smooth muscle cells and glial cells	Activation of PDGF receptor tyrosine kinase, phosphatidyl inositol turnover, proliferation of mesenchymal and smooth muscle cells.
PDGF(BB) human, recombinant	25kDa BB homodimer, 218aa 15kDa subunit	*c-sis*	Mammalian and avian cells of mesodermal and neuroectodermal origin such as fibroblasts, smooth muscle cells and glial cells	Activation of PDGF receptor tyrosine kinase, phosphatidyl inositol turnover, proliferation of mesenchymal and smooth muscle cells.
TGF-α human, recombinant	5.5kDa, 50aa	Sarcoma growth factor	Endoderm and ectoderm derived cells such as mammary, cornea, epidermis, liver, pancreas, nerve, glial, amniotic and adrenal medulla cells	Activation of EGF receptor tyrosine kinase and protein kinase C, modulation of proliferation in a variety of cell types.
TGF-β	25kDa dimer, composed of β and/or β_2 subunits	CIF-A, TIF-1, TGF-β1	Fibroblasts, enothelial cells, epithelial cells, T cells, B cells	Mediation of inflammation and injury repair, T cell and B cell proliferation, chemotaxis of fibroblasts at low factor concentrations, inhibition of proliferation at higher concentrations. Stimulates anchorage-independent growth of fibroblasts. Unknown signal transduction pathway.

Cellular Regulation

I. Properties and Applications of Growth Factors and Cytokines

(continued)

Serum-Free, Clonal Density Cell Proliferation Assays of Growth Factors

The assays typically used for growth factors record the factor-dependent proliferation of a target cell population, frequently measured as incorporation of [^{3}H]thymidine. Often, assays to quantify growth factor bioactivity are not easily reproduced in different laboratories. However, Promega has developed a series of easily reproduced quality assurance assays to document the biological activity of our line of growth factors, and to assist our customers with measuring growth factor bioactivity in their laboratories. Our strategy is to use established serum-free clonal density cell proliferation assays whenever possible (9) to provide a defined set of conditions for measurement of growth factor activity.

Serum is an incompletely defined mixture containing many factors that alter cell growth. Different lots of serum contain significant and variable quantities of growth promoting and attachment factors, including PDGF, insulin, IGF-I, IGF-II, EGF, and fibronectin, to name a few. Serum also contains growth-inhibiting substances such as TGF-β as well as growth factor binding proteins that can alter the bioactivity of factors such as IGF-I, IGF-II, and PDGF. When exogenous factors are added to serum-supplemented medium, their mitogenic activity reflects only the concentrations remaining free of binding proteins and able to interact with the cell surface receptor. The consequence is that serum can obscure the interpretation of results from growth factor assays.

The Balb/c 3T3 cell line is used for many of the assays because: 1) stock cultures are easily maintained, 2) it is safe to use, 3) a serum-free medium has been developed to support growth, 4) it is multi-factor responsive (thus the same target cell line can be used to assay for numerous growth factors including IGF-I, IGF-II, bFGF, aFGF, EGF, TGF-α, and PDGFs), and 5) the cell line is readily available to all cell culture laboratories, and thus the assays can be easily reproduced by our customers.

Choosing the proper cell density is an important consideration when designing growth factor assays. Cells grown *in vitro* are known to release various autocrine factors into the culture medium (a process known as conditioning) which can affect their own growth. This phenomenon can occur in serum-supplemented or serum-free media, resulting in the accumulation of factors which may confuse the interpretation of experimental results. Accumulation of autocrine factors can be minimized if proliferation assays are done at low (clonal) cell density.

One method used by Promega to measure growth factor activity is the cell number assay. Although cell number assays are generally more time consuming and labor intensive than [^{3}H]thymidine incorporation assays, they offer the most direct measurement of cell growth and provide an accurate determination of the number of biological activity units (ED_{50}) of growth factors. Counting the number of viable cells over the course of an experiment avoids possible artifacts (such as alternative pathways of pyrimidine metabolism) associated with measuring [^{3}H]thymidine incorporation.

Another, more convenient, cell proliferation assay is the MTT assay. Mitochondrial dehydrogenases convert MTT (dimethylthiazol-2-yl-diphenyltetrazolium bromide) into a colored product that can be correlated with cell number. The assay is nonradioactive, inexpensive, and requires a minimum of labor becaue it can be performed in a 96 well format and read with an automated plate reader.

It should be noted that the ED_{50} value may change depending on a variety of assay parameters including: 1) the type of assay done (i.e., cell number, [^{3}H]thymidine incorporation, or dye reduction), 2) the cell line used for the assay, 3) the medium used to support growth, and 4) the length of the assay.

II. Properties and Applications of Protein Kinases

The phosphorylation of proteins by protein kinases is an important mediator of cell metabolism. Protein kinase activity has been shown to be involved in regulation of intercellular communication, intracellular signal transduction, cell division, protein activity and several other cellular systems (6,11). The number of known mammalian protein kinases has been rapidly increasing and may eventually number over one thousand (11). Certain kinases, such as cAMP-dependent protein kinase and protein kinase C, have thus far been shown to be ubiquitous in mammalian tissue (12).

Two general types of protein kinases have been identified, distinguished by the amino acid residues which they phosphorylate: serine/threonine kinases and tyrosine kinases. Most of the serine/threonine kinases studied to date are soluble, cytosolic proteins (12). This group includes protein kinase C (PKC), cAMP-dependent protein kinase (PKA), and cGMP-dependent protein kinase (PKG). In contrast, tyrosine kinase activities are often found in the cytosolic regions of membrane-associated receptors. In many cases, such as the EGF receptor and the insulin receptor, ligand binding to the receptor's extracellular domain leads to activation of the tyrosine kinase activity (13,14). This results in phosphorylation of the receptor as well as other intracellular targets, initiating a cascade of signal transduction.

Because protein phosphorylation is known to be involved in so many cellular pathways, it is important to determine if protein phosphorylation plays a regulatory role in a novel system under study. This question may be addressed using protein kinase activators or inhibitors or, alternatively, by using purified kinases.

Specific kinase activators or inhibitors may be used *in vivo* or *in vitro*. An observed effect on physiology or phosphorylation in the system under study suggests the involvement of the relevant protein kinase(s). Purified kinases can be added to *in vivo* systems by microinjection (15), and their effects noted. This approach is particularly informative when using PKA, because the active catalytic subunit may be added together with or separately from the regulatory (inhibitory) subunit. The role of PKA in a given response can be confirmed by demonstrating that the presence of the regulatory subunit inhibits the response, but that cAMP reverses this inhibition.

In *in vitro* systems, purified protein kinases are useful for determining if a target protein may be phosphorylated by a specific kinase. In brief, this is performed by adding the kinase, its activators, and radiolabeled ATP to the *in vitro* system. Proteins labeled by ^{32}P then are detected by a standard kinase assay (16), by autoradiography after gel electrophoresis, or in some cases by an immunoblot assay (17). To identify the amino acid residue phosphorylated by a given kinase, the protein is ^{32}P-labeled in the presence of the kinase and then acid-digested. The amino acids generated are separated by thin layer chromatography and the migration of the labeled amino acid is compared with radiolabeled standards of phosphoserine, phosphothreonine and phosphotyrosine (18).

If the amino acid sequence of a putative substrate is known, it is possible to deduce potential phosphorylation sites. Protein kinases preferentially phosphorylate discreet sequence motifs, most of which are known (19). If a protein is known to be a substrate for a particular kinase, the number and location of the phosphorylated residues can be determined by epitope mapping using Promega's Probe-Design™ peptide separation system and related products (20). The target protein is radiolabeled by incubation with activated kinase and radiolabeled ATP. The protein then is digested with proteases to yield discreet fragments, which are separated by SDS gel electrophoresis and transferred to a PVDF membrane. The ^{32}P-labeled fragments are identified by autoradiography and then sequenced directly from the PVDF support. If the primary sequence of the target protein is known, the amino acid sequence surrounding each phosphorylation site can be used to map these sites on the protein. The precise location of phosphorylation within each proteolytic fragment can be inferred from the preferred recognition sequence motifs of the kinase used.

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Table 3. Properties of Protein Kinases. Several properties of the better-studied eukaryotic protein kinases are listed below. The phosphorylation target sequences are given using the one-letter symbols for amino acids. Kinases listed in boldface type are available from Promega.

Name	Phosphorylation Targets/ Preferred Recognition Motif	Molecular Structure	Activators & Inhibitors	Tissue Sources	*In vitro* Substrates
cAMP-dependent protein kinase	Ser/Thr XRRXS*X	R_2C_2 tetramer **40kDa catalytic subunit** (21) **51-54kDa regulatory subunit**	Activators: cAMP (4 sites per holoenzyme) Inhibitors: Walsh inhibitor, staurosporine (22), KT5720 (23)	Ubiquitous in eukaryotes	Autophosphorylates (21), histone H1 (24), synapsin 1 (25), MAP-2 (26), voltage-sensitive Na^+ channel, nicotinic acetylcholine receptor, β-adrenergic receptor, tyrosine hydroxylase, neurofilaments, myelin basic protein, guanylate cyclase, cyclic nucleotide-dependent phosphodiesterase, GABA-modulin
cGMP-dependent protein kinase	Ser/Thr XS*RX	Covalently bound homodimer, 76kDa subunits (21)	Activator: cGMP (2 sites per holoenzyme) Inhibitor: KT5823	Smooth muscle, lung, aorta, cerebellum	Autophosphorylates (27), histone H1 (24), G-substrate (24)
Protein kinase C	Ser/Thr XRXXS*XRX	82kDa monomer	Activators: Ca^{+2}, phosphatidyl serine, phorbol esters Inhibitors: Staurosporine (28), calphostin C (23)	Ubiquitous in eukaryotes	Autophosphorylates , histone H1 (24), EGF receptor, phospholambin, vinculin, eIF-2, troponin T, β-adrenergic receptor, glycogen synthase, synapsin 1, MAP-2 (26), tyrosine hydroxylase, nicotinic acetylcholine receptor, myelin basic protein, smooth muscle myosin light chain, postsynaptic glycine receptor (29), neurogranin (30)
Calmodulin-dependent protein kinase II	Ser/Thr	550-650kDa, mixture of 50kDa α subunits and 60kDa β subunits	Activators: Ca^{+2}, calmodulin Inhibitors: Phenylglyoxal	Enriched in brain, muscle liver	Autophosphorylates (31), syntide (32), glycogen synthase (24), tyrosine hydroxylase, myelin basic protein, smooth muscle myosin light chain, ribosomal protein S6, MAP-2 (26)
Epidermal growth factor receptor	Tyr TAENAEY*LRVAP	170kDa membrane-bound monomer (33)	Activators: EGF, TGF-α, vaccinia virus growth factor (33) Inhibitor: Tyrphosten (34)	Most eukaryotic tissues	Autophosphorylates (6), phospholipase C-γ, lipocortin-1
Insulin receptor	Tyr TRDIY*ETDY*Y*RK	$\alpha_2\beta_2$ membrane-bound tetramer, 125-135kDa α subunits, 95kDa β subunits (35)	Activator: Insulin	Many sources, including placenta, liver, hepatoma, and lymphocytes (35)	Autophosphorylates (6), MAP-2, calmodulin-dependent protein kinase, calmodulin, tubulin (36), casein, histones (35)

Key to One-Letter Symbols Used:

A Alanine
D Aspartic Acid
E Glutamic Acid
R Arginine
K Lysine
I Isoleucine
L Leucine
N Asparagine
S Serine
T Threonine
V Valine
X Any Amino Acid
Y Tyrosine
* Site of Phosphorylation

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IV. Additional Cellular Regulation Literature Available from Promega

Growth Factor Product Guide

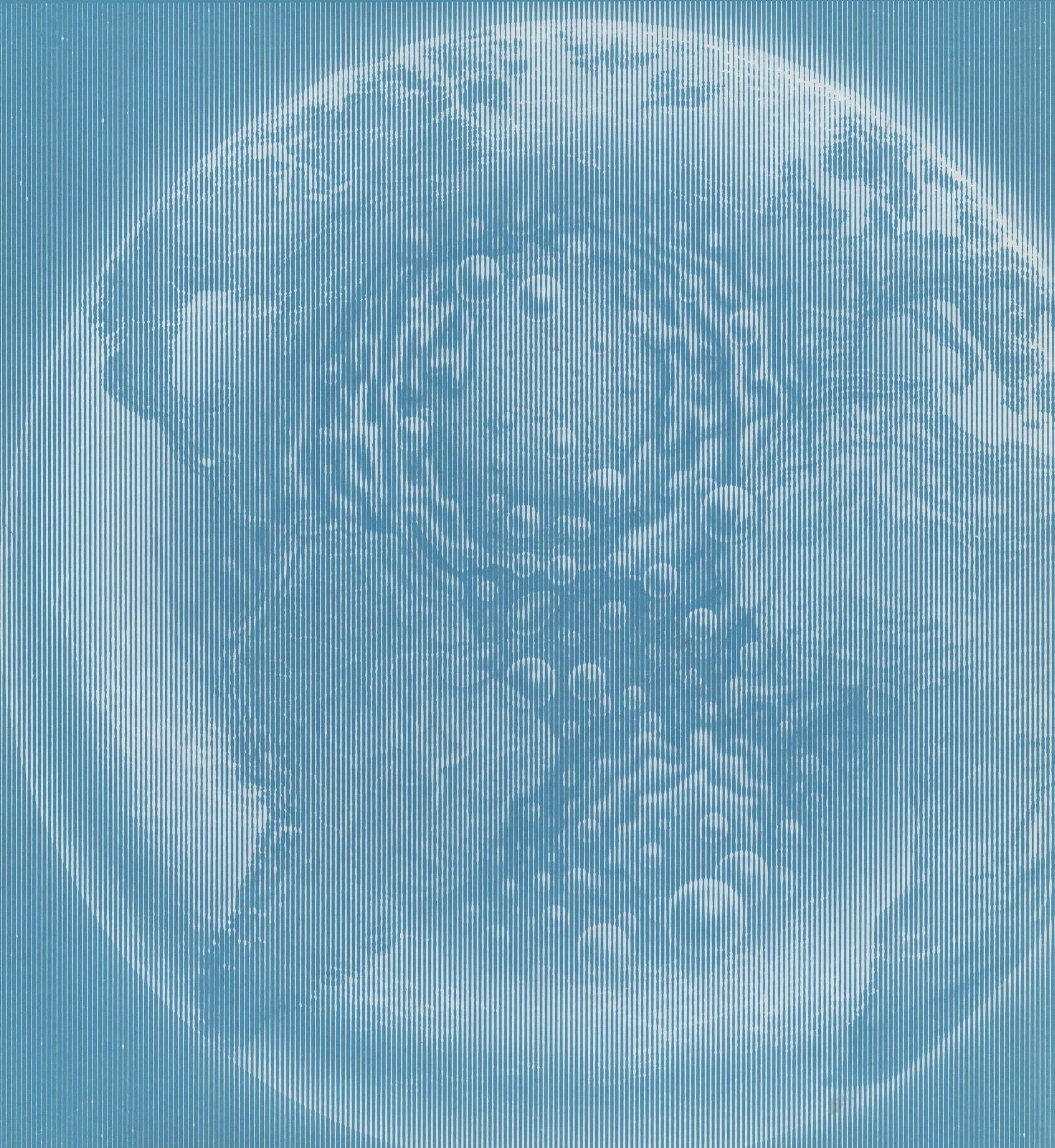
Promega Notes Articles

Issue	Title
27	Quality control assays for growth factor bioactivity

Notes

Promega

PROTOCOLS AND APPLICATIONS GUIDE



Eukaryotic Gene Regulation

Contents

Eukaryotic Gene Regulation

I. Strategies for Analysis of Eukaryotic Gene Regulation

Many eukaryotic protein-coding genes are regulated at the level of transcription. In these cases, the amount of an individual species of mRNA in the cell is determined primarily by the rate of initiation from the transcription unit which gives rise to the message. The term *transcription unit* is broadly defined to include all of the DNA sequences which correspond to the precursor mRNA (leader sequences, introns and exons) and which control the rate and initiation site of mRNA synthesis (promoter, initiator, activator and enhancer sequences).

The identification of transcriptional control elements relies upon a combination of DNA mutagenesis with *in vivo* and *in vitro* transcription assays. This kind of analysis typically uncovers multiple, discrete genetic elements, each consisting of several consecutive nucleotides, whose mutation severely limits the amount of mRNA a gene expresses. (Examples of such elements are shown in Figure 1.) One cannot gain a complete understanding of the molecular interactions driving gene expression, however, without a description of the entire transcription complex. The proteins

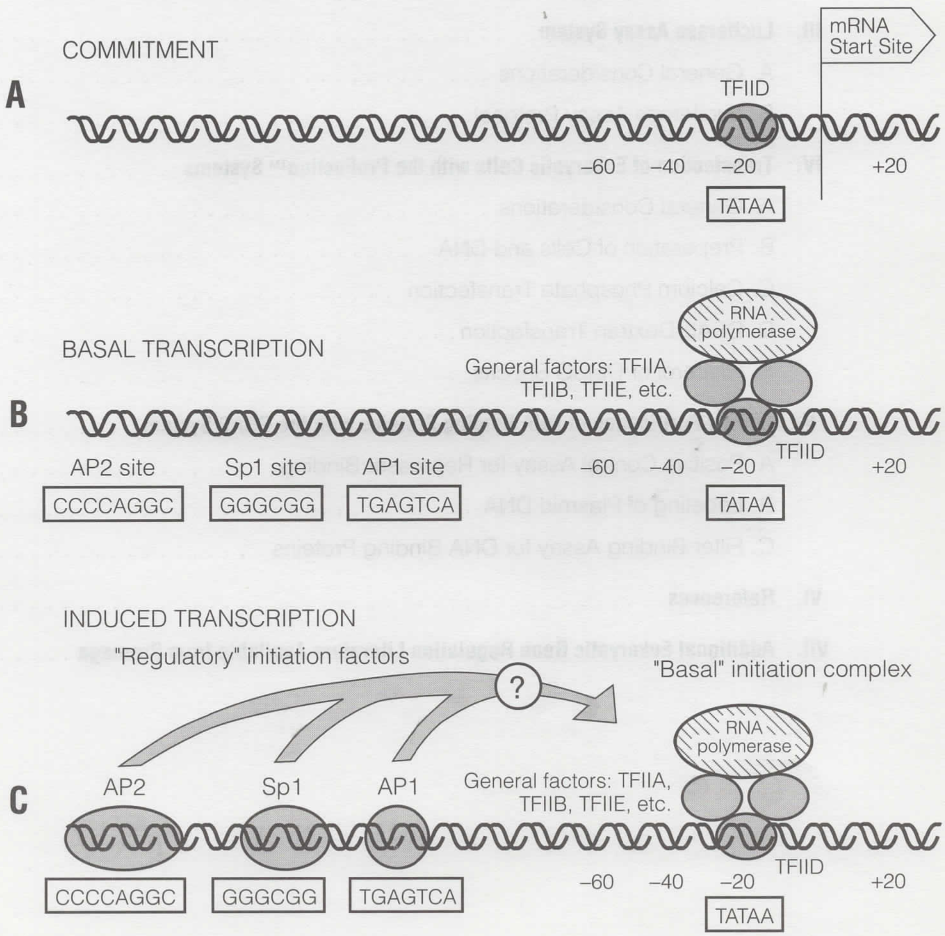


Figure 1. Schematic diagram of a generalized eukaryotic promoter complex. This figure is used, with modifications, by permission of B. Lewin (1).

I. Strategies for Analysis of Eukaryotic Gene Regulation

(continued)

which specifically bind the genetic control elements and whose interactions modulate the transit of RNA polymerase II across the gene define a unique transcription complex.

For many genes transcribed by RNA polymerase II, the critical protein that nucleates the assembly of the basal transcription complex is TFIID, the TATA box binding protein. As reviewed by Lewin (1), this binding step may be viewed as a prerequisite, or commitment step, in the formation of the remainder of the complex. Figure 1 illustrates the steps of assembling a basal and an induced (activated or regulated) transcription complex after binding of TFIID.

While it seems relatively clear that a number of general transcription factors assemble after TFIID to form the basal transcription complex (Figure 1B), current data are unable to explain how upstream factors, which bind to genetically defined control elements, interact with the basal apparatus to increase the rate of mRNA synthesis (Figure 1C). Most proposals favor the hypothesis that long distance interactions influence the basal complex by bringing upstream factors close to the start site of mRNA synthesis. Many current studies view the complex assembled at the TATA box as the critical recognition structure for RNA polymerase II, which by itself can not initiate specific mRNA synthesis. According to this view, domains of TFIID may be involved in mediating the changes that factor-factor interactions bring to the transcription complex.

Solving the nature of these changes *in vitro* requires two important tools: reliable and active nuclear extracts (Figure 2) and purified, recombinant transcription factors. Promega supplies HeLa and *Drosophila* nuclear extracts, transcription factors (including SP1, AP1, AP2 and TFIID), and a variety of kits used for analysis of eukaryotic promoter elements.

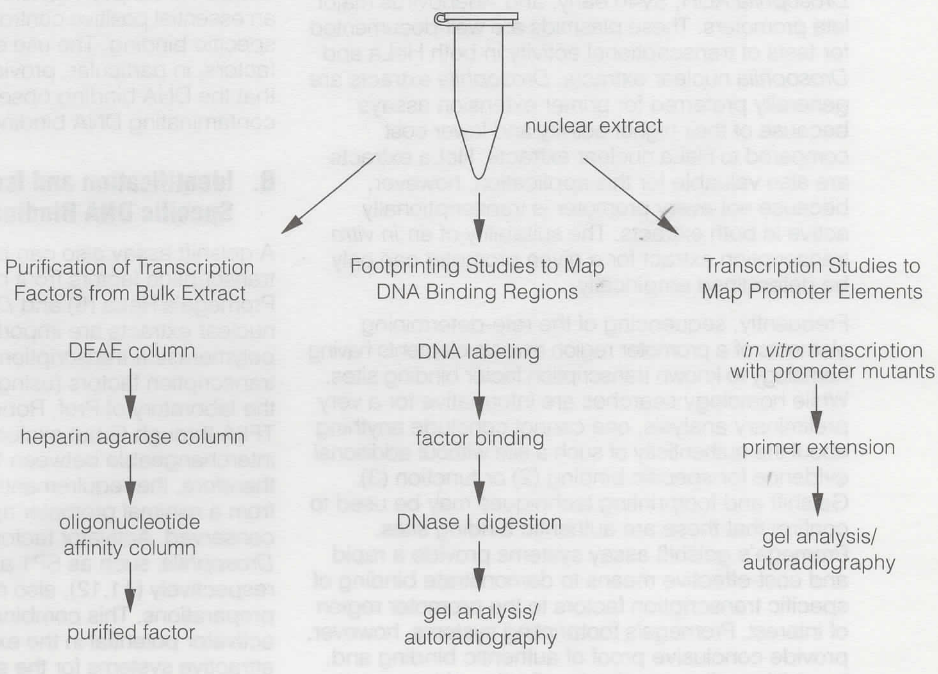


Figure 2. Applications of nuclear extracts for studies of eukaryotic transcription.

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(continued)

A. Identification of Binding Sites for Eukaryotic Transcriptional Regulatory Proteins

The key elements of a cloned promoter region are typically mapped by introduction of specific mutations followed by analysis of the effects on transcription using a primer extension assay. *Drosophila* nuclear extracts are used to synthesize RNA transcripts from the normal and mutated templates (Figure 2). To assay the levels of specific transcription by primer extension, cDNA is synthesized from these transcripts using an end-labeled primer homologous to the target gene. The amount of cDNA obtained is a measure of the level of specific transcription, and the size of the cDNA can serve to identify altered transcription start sites which may result from promoter mutations. The transcriptional activity of new or uncharacterized promoters typically is compared to that of known promoters. Promega provides the *Drosophila* segmentation gene, *Krüppel*, as a positive control promoter plasmid. Three additional promoter plasmids are also available, containing the *Drosophila* ADH, SV40 early, and Adenovirus major late promoters. These plasmids are well-documented for tests of transcriptional activity in both HeLa and *Drosophila* nuclear extracts. *Drosophila* extracts are generally preferred for primer extension assays because of their higher activity and lower cost compared to HeLa nuclear extracts. HeLa extracts are also valuable for this application, however, because not every promoter is transcriptionally active in both extracts. The suitability of an *in vitro* transcription extract for a given promoter can only be determined empirically.

Frequently, sequencing of the rate-determining elements of a promoter region reveals elements having homology to known transcription factor binding sites. While homology searches are informative for a very preliminary analysis, one cannot conclude anything about the authenticity of such a site without additional evidence for specific binding (2) or function (3). Gelshift and footprinting techniques may be used to confirm that these are authentic binding sites. Promega's gelshift assay systems provide a rapid and cost-effective means to demonstrate binding of specific transcription factors to the promoter region of interest. Promega's footprinting systems, however, provide conclusive proof of authentic binding and, in addition, allow precise localization of transcription factor binding sites. Most importantly, footprinting allows one to determine whether or not every rate-determining promoter element corresponds to a factor binding site.

The gelshift assay procedure is simple and straightforward. An end-labeled, double-stranded DNA fragment containing the promoter sequence is mixed with a nuclear extract and then subjected to gel electrophoresis. Nuclear proteins that bind to the promoter region cause a retardation in migration of the labeled fragment. The identities of bound factors are inferred by competition with oligonucleotides containing consensus binding sites for specific transcription factors.

To perform a footprinting assay, an end-labeled double-stranded DNA fragment containing the promoter sequence is bound to a specific transcription factor. The protein-DNA complex then is partially digested with DNase I. DNA regions protected by protein binding are spared from digestion, and are mapped by electrophoresis on a high resolution DNA sequencing gel. Specific blocks of nucleotides protected from digestion correspond to regions contacted by DNA binding domains of the transcription factor used. The inclusion of highly purified, cloned transcription factors in these systems provides an essential positive control for demonstrating factor-specific binding. The use of cloned transcription factors, in particular, provides absolute certainty that the DNA binding observed is not due to a contaminating DNA binding activity.

B. Identification and Isolation of Sequence-Specific DNA Binding Proteins

A gelshift assay also can be used to identify novel transcription factors from nuclear extracts (4). Promega's HeLa (5) and *Drosophila* embryo (6,7) nuclear extracts are important sources of RNA polymerase II transcription factors. The general transcription factors (using the nomenclature of the laboratory of Prof. Robert Roeder), including TFIIA through G but excluding C (8,9), appear interchangeable between these two systems (10); therefore, the requirements for basal transcription from a minimal promoter appear to be functionally conserved. Activator factors unique to HeLa and *Drosophila*, such as SP1 and GAGA factor, respectively (11,12), also may be found in these preparations. This combination of basal and activator potential in the extracts makes them attractive systems for the study of general and regulated transcription complexes.

Once sequence-specific DNA binding proteins have been identified, they may be isolated by the DNA affinity chromatography technique of Kadonaga and

Promega

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(continued)

Tjian (13), in which DNA binding proteins are purified by chromatography on heparin agarose and oligonucleotide columns. Substantial discussion of this technique may be found in references 14 and 15. Promega offers transcription factor-qualified heparin agarose resins for the starting points of such affinity chromatography purifications, and our experience with oligonucleotide synthesis and large-scale affinity chromatography may be helpful in designing your protein purification protocols.

For more information and complete protocols for the use of Promega's *in vitro* transcription, primer extension, gelshift and footprinting systems, please contact our Technical Services department.

C. Reporter Gene Systems

To study the effects *in vivo* of putative promoters or enhancers, these sequences can be linked to a reporter gene and transfected into eukaryotic cells. Such reporter gene systems are able to provide quantitation of transcription in whole cells in response to transcriptional activators. Reporter gene systems also are well suited for identification of enhancer elements, since enhancer sequences are not necessarily contiguous with the target gene and are identified primarily by their effects on transcriptional activity. Promega's CAT (chloramphenicol acetyltransferase) reporter system (pg. 286) contains a series of four plasmids which, together, allow the researcher to identify putative promoter and enhancer elements in eukaryotic genomic DNA sequences.

The transcription level measured for a reporter gene is highly dependent on the efficiency of transfection obtained with the target cells. Variation of transfection efficiency may be controlled for by using a control plasmid transfected into the target cells at the same time as the test plasmid. The pSV-β-Galactosidase control plasmid (pg. 290) is designed for this purpose. Assays for CAT and β-galactosidase enzyme activity are described on pgs. 288-293. Two other widely used reporter genes are growth hormone and luciferase. Growth hormone is secreted from cells and monitored in the medium by radioimmunoassay (16).

The gene encoding firefly luciferase has proven to be highly effective as a reporter gene because the assay of enzyme activity is extremely sensitive, rapid, easy to perform and is relatively inexpensive (17,18,19). Additional advantages to the use of luciferase as a reporter enzyme are that it is a single polypeptide (62kDa) that requires no post-translational modifications for activity (20,21) and that it exhibits no endogenous activity in mammalian cells. Also, light production by luciferase has the highest efficiency known of any chemiluminescent reaction (22). Promega's luciferase assay system, described in detail in Section III (pg. 294), is a substantial improvement over conventional methods in terms of both sensitivity and simplicity (23).

II. pCAT® Reporter Gene Systems

The CAT reporter system offers a sensitive and rapid means to perform *in vivo* analysis of eukaryotic transcriptional regulatory sequences (24). With this system, genomic DNA fragments containing putative promoter or enhancer sequences can be linked to the chloramphenicol acetyltransferase (CAT) reporter gene in specially constructed plasmids. After transfection of these plasmids into cultured cells, transcriptional effects of the putative promoter or enhancer sequence are measured by the activity of the CAT gene in crude cell extracts. Since CAT is a bacterial gene, levels of CAT enzyme activity can be quickly and easily assayed with little or no background from endogenous cellular gene activity.

A typical test for putative regulatory sequences is straightforward: the genomic DNA fragment of interest is ligated with the desired pCAT plasmid and the recombinant plasmid is then transfected into a eukaryotic cell line (Section IV.C and IV.D, pgs. 301-303) by standard techniques such as calcium phosphate-DNA coprecipitation (25,26), DEAE-dextran (25,27), or electroporation (26). After two days, the cells are harvested, lysed and the cell extracts are assayed for CAT activity. Two assays for CAT activity are described in Section II.C, pg. 288.

II. pCAT® Reporter Gene Systems

(continued)

A. pCAT Reporter Plasmids

Promega's CAT reporter system consists of four plasmids which express CAT activity in eukaryotic cells and two optimized assay systems for measurement of CAT activity.

pCAT® Plasmids

The four pCAT plasmids (Figure 3), originally designed by Dr. Nadia Rosenthal (27), allow the researcher to assay genomic DNA fragments for promoter or enhancer sequences separately or together. All four plasmids have a common plasmid context, meaning that they share a common pUC19 backbone. The pCAT-Basic, pCAT-Enhancer, and pCAT-Promoter plasmids have been designed and tested to give extremely low background levels of CAT transcription in several eukaryotic cell lines, including HeLa and NIH 3T3.

Note: We do not recommend the use of COS cells with the pCAT vectors. COS cells, as a result of being transformed with portions of the SV40 virus, contain the large T antigen which functions in SV40 replication. Replication of the pCAT vectors containing the SV40 origin of replication produces high background levels of the CAT enzyme in transfected COS cells.

pCAT®-Basic

The pCAT-Basic plasmid lacks eukaryotic promoter and enhancer sequences. This allows the researcher maximum flexibility in cloning any putative regulatory sequences into the convenient multiple cloning sites. Expression of CAT activity in cells transfected with this plasmid is dependent on insertion of a functional promoter upstream from the CAT gene. Enhancer elements can be inserted upstream from the promoter or at the *Bam*H I site downstream from the CAT gene.

pCAT®-Enhancer

The pCAT-Enhancer plasmid contains an SV40 enhancer element built into the plasmid backbone. This allows verification of functional promoter-CAT junctions when testing putative promoter sequences, since presence of the enhancer in many cases will result in transcription of the CAT gene at high levels.

pCAT®-Promoter

The pCAT-Promoter plasmid contains an SV40 promoter upstream from the CAT gene. Genomic DNA fragments containing putative enhancer elements can be inserted in either orientation, and upstream or downstream from the promoter-CAT transcriptional unit.

pCAT®-Control

The pCAT-Control plasmid contains SV40 promoter and enhancer sequences, resulting in strong expression of CAT activity in many types of eukaryotic cells. This plasmid is useful for monitoring transfection efficiency in general and is a convenient internal standard for comparing promoter and enhancer strengths. The pCAT-Control plasmid, containing both SV40 promoter and enhancer sequences, exhibits strong expression of the CAT gene in many cell lines.

B. Preparation of Cell Extracts

Clone the DNA fragment of interest into the desired pCAT vector. Refer to the Plasmid Cloning and Transcription chapter (pgs. 51-57) for general information on plasmid cloning. The pCAT vectors are supplied with the bacterial strain HB101. Transfect the recombinant plasmid into the desired eukaryotic cell line by standard techniques such as calcium phosphate DNA coprecipitation (25,26), DEAE-Dextran (25,27) or electroporation (26). Detailed transfection protocols are provided in Section IV (pg. 297). For CAT transient expression assays, cell extracts are typically prepared 48-72 hours post-transfection. Two alternative methods are described below for extract preparation from adherent cells grown in tissue culture.

1. Tris Buffer Extract (27)

Reagents Needed

- PBS buffer (pg. 293)
- TEN buffer (pg. 293)
- 0.25M Tris-HCl, pH 8.0

II. pCAT® Reporter Gene Systems

(continued)

Protocol

a. Wash the cells with PBS (Mg^{+2} and Ca^{+2} free) buffer (pg. 293) 5 times. Aspirate well after the final wash.

b. Add 1ml (per 100mm dish) of TEN buffer (pg. 293) and incubate the cells for 5 minutes at room temperature.

c. Scrape the cells with a rubber policeman and transfer them to a microcentrifuge tube.

d. Spin cells at 4°C for 10 minutes. Remove the supernatant and resuspend the pellet (vortex vigorously) in 150µl of 0.25M Tris-HCl, pH 8.0.

e. Subject the extract to 3 freeze-thaw cycles, with vortexing after each thaw cycle. (Freeze in dry ice and thaw at 37°C).

f. Heat the extract at 60°C for 10 minutes to inactivate endogenous acetylase. This heating step does not inactivate CAT.

g. Spin the extract for 10 minutes in a micro-centrifuge and transfer the supernatant to a fresh tube.

h. Store the extract at –70°C.

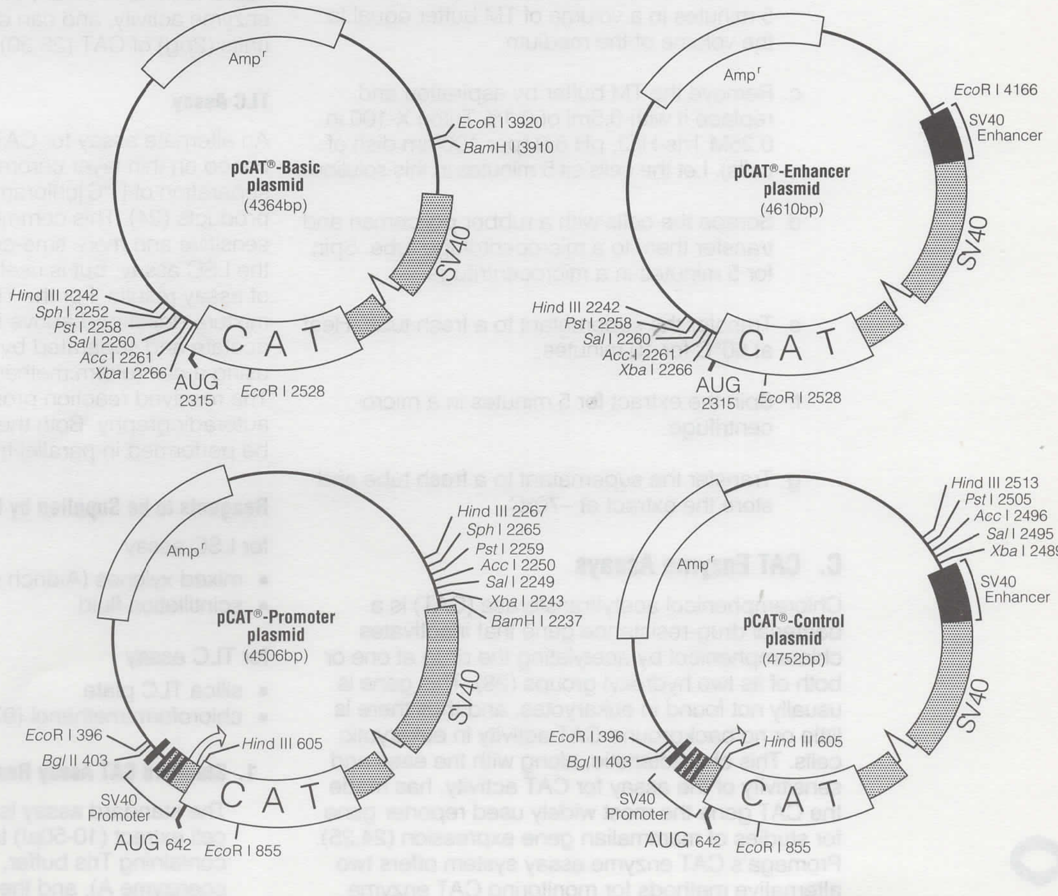

Figure 3. pCAT plasmid circle maps.

II. pCAT® Reporter Gene Systems

(continued)

2. Detergent Extract

This extract is quicker and easier to prepare than the Tris buffer extract. However, the final cell extracts are more dilute and the detergent interferes to a small extent (approximately 10%) with the liquid scintillation counting CAT activity assay.

Reagents Needed

- TM buffer (pg. 293)
- Triton extraction buffer (pg. 293)

Protocol

a. Wash the cells 2 times in TM buffer.

b. Incubate the cells at room temperature for 5 minutes in a volume of TM buffer equal to the volume of the medium.

c. Remove the TM buffer by aspiration and replace it with 0.5ml of 0.1% Triton X-100 in 0.25M Tris-HCl, pH 8.0 (per 100mm dish of cells). Let the cells sit 5 minutes in this solution.

d. Scrape the cells with a rubber policeman and transfer them to a microcentrifuge tube. Spin for 5 minutes in a microcentrifuge.

e. Transfer the supernatant to a fresh tube. Heat at 60°C for 10 minutes.

f. Spin the extract for 5 minutes in a microcentrifuge.

g. Transfer the supernatant to a fresh tube and store the extract at –70°C.

C. CAT Enzyme Assays

Chloramphenicol acetyltransferase (CAT) is a bacterial drug-resistance gene that inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups (28). This gene is usually not found in eukaryotes, and thus there is little or no background CAT activity in eukaryotic cells. This characteristic, along with the ease and sensitivity of the assay for CAT activity, has made the CAT gene the most widely used reporter gene for studies of mammalian gene expression (24,25). Promega's CAT enzyme assay system offers two alternative methods for monitoring CAT enzyme activity in transfected cells.

LSC Assay

The more rapid, sensitive, and convenient of these two assays is based on liquid scintillation counting (LSC) of CAT reaction products. Cell extracts are incubated in a reaction mix containing ^{14}C- or ^{3}H-labeled chloramphenicol and n-butyryl coenzyme A. CAT transfers the n-butyryl moiety of the cofactor to chloramphenicol.

For the LSC assay, the reaction products are extracted with a small volume of xylene. The n-butyryl chloramphenicol products partition into the xylene phase while unmodifed chloramphenicol remains in the aqueous phase (29). After two brief back-extractions, a portion of the xylene phase is mixed with scintillant and counted in a scintillation counter. This assay can be completed in as little as 2-3 hours, is linear for nearly three orders of magnitude of enzyme activity, and can detect as little as 3×10^{-4} units (2pg) of CAT (28,30).

TLC Assay

An alternate assay for CAT enzymatic activity is based on thin layer chromatography (TLC) separation of [^{14}C]chloramphenicol substrate and products (24). This commonly used assay is less sensitive and more time-consuming to perform than the LSC assay, but is useful as a visual confirmation of assay results. For the TLC assay, the reaction mixture described above is extracted with ethyl acetate and separated by TLC on silica plates using a chloroform:methanol::97:3 solvent system. The resolved reaction products are detected by autoradiography. Both the LSC and TLC assays can be performed in parallel from a single cell extract.

Reagents to be Supplied by the User

for LSC assay

- mixed xylenes (Aldrich Cat.# 24-764-2)
- scintillation fluid

for TLC assay

- silica TLC plate
- chloroform:methanol (97:3)

1. Standard CAT Assay Reaction

The standard assay is performed by adding the cell extract (10-50µl) to a microcentrifuge tube containing Tris buffer, the cofactor (n-butyryl coenzyme A), and the radiolabeled substrate chloramphenicol, in a final volume of 125µl.

II. pCAT® Reporter Gene Systems

(continued)

a. Prepare the following reaction mixture in a 1.5ml microcentrifuge tube:

cell extract	10-50µl
0.25M Tris-HCl, pH 8.0	65-105µl
[^{14}C]chloramphenicol (at 0.025mCi/ml) (see Note 1)	1-10µl
n-butyryl coenzyme A (at 5mg/ml) (this will initiate the reaction)	5µl
final volume	125µl

Also prepare a standard curve of CAT activity consisting of an enzyme blank containing no cell extract and enzyme controls. Set up reactions with 0.1, 0.05, 0.01 and 0.005 units of CAT. Prepare appropriate dilutions of CAT in cold Tris buffer before the assay. For example, dilute 1µl of CAT enzyme at 10u/µl in a total of 500µl and then add 5µl of the diluted enzyme to the reaction mix for a total of 0.1u of CAT per reaction.

b. Incubate the reactions at 37°C for a fixed period of time (from 30 minutes to 20 hours).

Note: Kinetics of the reaction can be monitored by taking aliquots of a reaction mix at several time points during the first 60 minutes. This data provides an accurate measurement of enzyme activity, particularly in reactions containing high levels of enzyme activity. Longer overnight incubations give higher signals and greater sensitivity, and may be desirable with extracts containing low levels of CAT activity.

c. Briefly spin the tubes in a microcentrifuge.

d. Terminate the reactions by adding 300µl of mixed xylenes to each tube. Continue with the LSC assay instructions OR add 500µl of ethyl acetate and continue with the TLC assay instructions.

2. LSC Assay

a. Vortex with xylenes for 30 seconds. Centrifuge for 3 minutes to achieve good phase separation.

b. Transfer the entire upper phase (xylenes) to a fresh tube. Add 100µl of fresh 0.25M Tris buffer, pH 8.0 and back-extract by repeating Step 2.a.

c. For maximum sensitivity, back-extract a second time by transferring the xylenes again to a fresh tube and adding 100µl of 0.25M Tris buffer, pH 8.0. If maximum sensitivity is not required, proceed directly to Step 2.d.

d. Carefully remove 200µl of the upper, xylenes phase and transfer it to a scintillation vial.

e. Add an appropriate scintillation fluid (e.g., Ready Safe by Beckman) and count samples in a liquid scintillation counter.

f. The cpm measured in each sample represent the butyrylated chloramphenicol products. The chloramphenicol substrate partitions preferentially into the aqueous phase, and the multiple back-extractions reduce the amount of chloramphenicol present in the xylene phase.

3. TLC Assay

a. Vortex with ethyl acetate for 1 minute. Centrifuge for 3 minutes to achieve good phase separation.

b. Transfer the upper, organic phase to a fresh tube and evaporate to dryness.

c. Resuspend the residue in 30µl of ethyl acetate. Spot 10µl of each sample onto a silica gel TLC plate and dry.

d. Place the TLC plate in a tank pre-equilibrated for 1 hour with chloroform:methanol::97:3.

e. Run the silica plate for 1 to 1.5 hours in a closed tank, until the solvent is approximately halfway up the plate.

(continued on next page)

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(continued)

f. Remove the silica plate from the tank and dry it at room temperature.

g. Cover the plate with plastic wrap (e.g., Saran Wrap) and expose it to X-ray film for an autoradiogram.

h. Develop the autoradiogram in 2 to 7 days.

i. Chloramphenicol becomes butyrylated at two sites and these two forms migrate faster than the unmodified chloramphenicol substrate on the TLC plates in this solvent system. After autoradiography, both of the butyrylated products can be scraped from the plate and counted together in a scintillation counter for quantitation of CAT activity.

Notes:

1. The use of high specific activity [^{3}H]chloramphenicol requires the addition of unlabeled chloramphenicol to ensure an adequate supply of substrate in the CAT assay. Currently, [^{3}H]chloramphenicol is supplied in ethanol and the specific activity is approximately 1,000 times higher than the corresponding [^{14}C]chloramphenicol. To prepare the ^{3}H compound, first dilute the specific activity 1,000-fold. For example, 250µCi of [^{3}H]chloramphenicol (250µl) at 50Ci/mmole is 5×10^{-6} mmole. Add 5×10^{-3} mmole unlabeled chloramphenicol (MW=323.14) which is 32µl of chloramphenicol at 50mg/ml in ethanol. The resultant specific activity is 50mCi/mmole. Next, bring the volume to 500µl with ethanol (in this example, add 218µl ethanol) for a concentration of 250µCi/500µl. Prior to assay, dilute this ethanol stock of [^{3}H]chloramphenicol 20-fold with 0.25M Tris-HCl, pH 8.0. This final specific activity is 50mCi/mmole. Addition of 10µl, or 0.25µCi, to the CAT reaction mix with a final volume of 125µl results in a concentration of 40µM chloramphenicol.
2. If the background of ^{3}H in the xylene phase is high, pre-extract the [^{3}H]chloramphenicol in Tris buffer twice with an equal volume of xylenes.
3. [^{14}C]chloramphenicol rather than [^{3}H]chloramphenicol is recommended for TLC assays because of the shorter exposure time required for autoradiography.

D. β-Galactosidase Assay System and Cotransfection Control Plasmid

The enzyme β-galactosidase is widely used as a reporter molecule. Promega's pSV-β-Galactosidase positive control plasmid employs this enzyme for monitoring transfection efficiencies of mammalian cells (Figure 4). The SV40 early promoter and enhancer drive transcription of the bacterial *lacZ* gene which, in turn, is translated into the β-galactosidase enzyme. β-galactosidase is an excellent reporter enzyme (27,31) which can be assayed in cell extracts using Promega's β-galactosidase assay system (pgs. 291-292) or in fixed cells by *in situ* staining (pg. 293).

The pSV-β-Galactosidase control plasmid can be co-transfected with your DNA of interest. For example, co-transfection with CAT reporter gene vectors provides cell extracts that can be assayed for both CAT and β-galactosidase activities. The pSV-β-Galactosidase reporter vector acts as an internal control for transient expression assays. A negative control extract, prepared from mock-transfected cells, should also be assayed for the presence of endogenous β-galactosidase activity in cultured cells (27).

The pSV-β-Galactosidase control plasmid is a modification of pRSV-βGAL (32) with SV40 and pUC18 sequences substituted for RSV and pBR322 sequences.

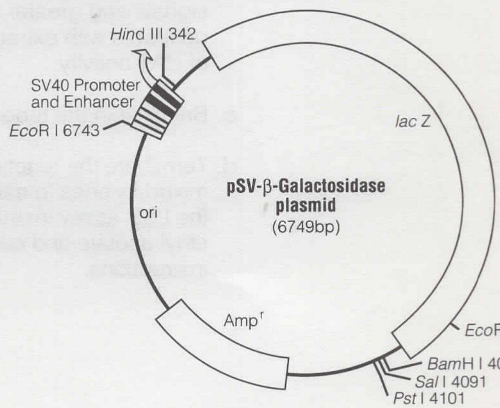

Figure 4. pSV-β-Galactosidase control plasmid circle map. The arrow indicates the direction of transcription of the *lacZ* gene.

II. pCAT® Reporter Gene Systems

(continued)

1. Transfection of Cells and Preparation of Cell Extract

The recommended amount of pSV-β-Galactosidase control plasmid to use for transfection of cells (60mm or 100mm dish) is 5-10μg. The optimal amount of plasmid DNA will be determined by the efficiency of transfection, which is very dependent upon the particular cell line and transfection protocol. Detailed transfection protocols are provided in Section IV (pg. 297). For transient expression assays, cell extracts are typically prepared 48-72 hours after transfection, using the freeze-thaw cell extraction protocol provided on pg. 286, Section B.1.

2. Standard β-Galactosidase Assay

Promega's β-galactosidase assay system provides a convenient method for assaying β-galactosidase activity in samples such as bacterial cell extracts or extracts prepared from cells transfected with the pSV-β-Galactosidase control plasmid DNA.

The standard assay is performed by adding a diluted sample to an equal volume of the assay 2X buffer which contains the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside). Samples are incubated until a yellow color is present and the reaction is then terminated. Absorbance at 420nm is read with a spectrophotometer (27).

Reagents Needed

- β-galactosidase assay 2X buffer (supplied, pg. 293)
- 1u/μl β-galactosidase in 0.1M sodium phosphate buffer, pH 7.3 (supplied)
- 1M sodium carbonate (supplied)

Protocol

a. Thaw the assay 2X buffer and mix well. If crystals are present in the 1M sodium carbonate, warm the sodium carbonate at 37°C to dissolve the crystals and then place at room temperature.

b. Prepare the following reaction mixtures in microcentrifuge tubes:

Positive Control

β-galactosidase assay 2X buffer	150μl
β-galactosidase (diluted on ice in water from the 1u/μl stock, prepared immediately before use)	0.05u
deionized water	to final volume 300μl

Note that this positive control reaction will turn yellow immediately. Alternatively, a β-galactosidase standard curve may be prepared to allow quantitation of activity in the sample. For a 30 minute reaction, a convenient range is 0 to 5 x 10^{-3} units of β-galactosidase.

Negative Control

β-galactosidase assay 2X buffer	150μl
non-transfected cell extract	(generally in a volume of 30μl)
deionized water	to final volume 300μl

Sample Reaction

β-galactosidase assay 2X buffer	150μl
transfected cell extract	(generally in a volume of 30μl)
deionized water	to final volume 300μl

Note: When working with small sample volumes, it may be convenient to first dilute the assay 2X buffer 2-fold with water, pipet 300μl of the assay 1X buffer into each tube, and then add sample to each tube.

c. Mix all samples by vortexing.

d. Incubate the reactions at 37°C for a fixed period of time until a yellow color is present, usually within 30 minutes. The incubations may be performed as long as 3 hours, providing that the reaction tubes are tightly capped.

e. Stop the reactions by adding 500μl of 1M sodium carbonate. Mix by vortexing.

f. Read the absorbance at 420nm.

Note: The standard reaction temperature for β-galactosidase is 37°C. However, for convenience, the reactions may be carried out at room temperature.

Eukaryotic Gene Regulation

II. pCAT® Reporter Gene Systems

(continued)

3. β-Galactosidase Microtiter Plate Assay

When many samples are to be tested, the β-galactosidase assay may be performed in a microtiter plate and read by a plate reader.

a. Thaw the assay 2X buffer and mix well. If crystals are present in the 1M sodium carbonate, warm the sodium carbonate at 37°C to dissolve the crystals and then place at room temperature.

b. Add the following reaction mixtures directly to the microtiter wells:

Positive Control

β-galactosidase assay 2X buffer	50µl
β-galactosidase (diluted on ice in water from the 1u/µl stock, prepared immediately before use)	0.025u
deionized water	to final volume 100µl

Note that this positive control reaction will turn yellow immediately. Alternatively, a β-galactosidase standard curve may be prepared to allow quantitation of activity in the sample. For a 30 minute reaction, a convenient range is 0 to 2 x 10^{-3} units of β-galactosidase.

Negative Control

β-galactosidase assay 2X buffer	50µl
non-transfected cell extract	(generally in a volume of 10µl)
deionized water	to final volume 100µl

Sample Reaction

β-galactosidase assay 2X buffer	50µl
transfected cell extract	(generally in a volume of 10µl)
deionized water	to final volume 100µl

Note: When working with small sample volumes, it may be convenient to first dilute the assay 2X buffer 2-fold with water, pipet 100µl of assay 1X buffer into each well, and then add sample to each well.

c. Mix all samples by pipetting well contents up and down.

d. Incubate the reactions at 37°C (see Note 5) for a fixed period of time until a yellow color is present (from 30 minutes to overnight).

e. Stop the reaction by adding 150µl of 1M sodium carbonate. Mix by pipetting the contents of each well up and down.

Notes:

a. The microtiter assay above is configured for a microtiter plate which has a maximum well volume of approximately 300µl. For microtiter plates with different maximum well volumes, the above reaction may be scaled up or down proportionally.

b. Plate readers generally perform best using microtiter plates which have flat-bottomed, optically clear wells.

c. The coatings applied to some microtiter plates may inhibit the β-galactosidase reaction. To test for this possibility, perform identical reactions in microtiter wells and in microcentrifuge tubes. Stop the reactions, pipet the tube reactions into the plate wells and read all samples in a plate reader. The absorbance values should be the same for both types of samples.

d. Many plate readers are limited in the number of wavelengths at which they can read. Although the peak absorbance of the products of the β-galactosidase/ONPG reaction is near 420nm, other wavelengths close to 420nm may be used to monitor the reaction.

e. The standard reaction temperature for β-galactosidase is 37°C. However, for convenience, the reactions may be carried out at room temperature.

II. pCAT® Reporter Gene Systems

(continued)

4. *In situ* Staining of Cells for β-Galactosidase Activity

Cells transfected with the pSV-β-Galactosidase control plasmid and expressing the β-galactosidase enzyme can be visualized under the microscope. The cells appear blue after being fixed and incubated with the substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) (33).

Reagents to be Supplied by the User

- PBS buffer
- glutaraldehyde solution
- X-Gal solution
- 70% glycerol

Protocol

a. Wash the cells 5 times with PBS buffer.

b. Fix the cells with glutaraldehyde solution (below) for 15 minutes.

c. Remove the glutaraldehyde solution.

d. Prepare X-Gal solution (below) and filter (0.2 micron). Filtration removes large crystals which may be present in the X-Gal solution.

e. Add the X-Gal solution to the cells and incubate at 37°C for 30-60 minutes.

f. Remove the X-Gal solution.

g. Cover the cells with a film of 70% glycerol.

h. View the cells with a phase contrast or light microscope.

Composition of Solutions

PBS buffer:

137mM	NaCl
2.7mM	KCl
4.3mM	Na_2HPO_4
1.4mM	KH_2PO_4

The final pH should be 7.1.

TEN buffer:

40mM	Tris-HCl, pH 7.5
1mM	EDTA
150mM	NaCl

TM buffer:

20mM	Tris-HCl, pH 7.5
2mM	$MgCl_2$

Triton extraction buffer:

0.1%	Triton X-100
0.25M	Tris-HCl, pH 8.0

β-Galactosidase assay 2X buffer:

200mM	sodium phosphate, pH 7.3
2mM	$MgCl_2$
100mM	β-mercaptoethanol
1.33mg/ml	ONPG

0.1M Sodium phosphate, pH 7.0:

60mM	Na_2HPO_4
40mM	NaH_2PO_4

Glutaraldehyde solution:

0.1M	sodium phosphate buffer, pH 7.0
1mM	$MgCl_2$

X-Gal solution:

0.2%	X-Gal
10mM	sodium phosphate, pH 7.0
1mM	$MgCl_2$
150mM	NaCl
3.3mM	$K_4Fe(CN)_6 \cdot 3H_2O$
3.3mM	$K_3Fe(CN)_6$

Eukaryotic Gene Regulation

III. Luciferase Assay System

A. General Considerations

The primary advantage of firefly luciferase as a genetic reporter is the extreme sensitivity of its assay (34). The assay also is rapid and easy to perform, does not use radioisotopes and is relatively inexpensive. The increased sensitivity especially aids in studies of low-level expression, such as that from weak promoters (35). In addition, the use of a luciferase reporter gene allows studies to be done using smaller amounts of biological materials or sooner following transfection of the reporter gene into target cells. Because endogenous luminescence is essentially absent from biological samples, there is no interference with the signal generated by luciferase.

Luciferase is a single polypeptide (62kDa) which requires no post-translational modifications for enzymatic activity. It contains no prosthetic groups, bound metals, nor disulfide bonds. Because of this structural simplicity, it can be expressed in virtually any host organism or cell type (19).

Promega's novel luciferase assay system* offers several advantages over conventional assays for luciferase (23). The reaction catalyzed by firefly luciferase is oxidation of beetle luciferin with concomitant production of a photon. Under conventional reaction conditions, the oxidation occurs from an enzyme intermediate, luciferyl-AMP. However, recent investigation has revealed that coenzyme A is a substrate in the luminescent reaction. In the presence of CoA, oxidation occurs through luciferyl-CoA. The result is light production without the characteristic self-inhibition of luciferase (36) observed in other assays.

The conventional assay for luciferase generates a "flash" of light that rapidly decays after enzyme and substrate are mixed, thus requiring automated injection luminometers for measurements of photon production. Promega's luciferase assay system allows for greater enzymatic turnover of luciferase (36), which results in greater light intensity that is nearly constant for measurements of up to several minutes (see Figure 5).

The constant light intensity generated in Promega's assay eliminates the need for rapid mixing protocols and automated injection devices. The simplified assay procedure is adaptable to different measurement methods for light production, such as scintillation counting or exposure to photographic film (10,11). Promega's luciferase assay system yields linearity over at least 8 decades of enzyme concentration, and less than 10^{-20} moles of luciferase have been detected under ideal conditions (23). Generally, users should achieve 100-fold greater sensitivity than with a chloramphenicol acetyltransferase (CAT) assay (34).

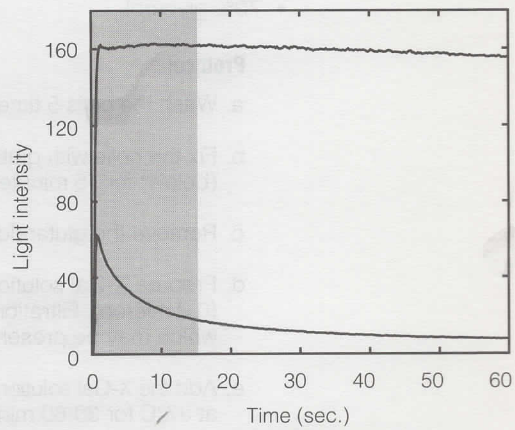

Figure 5. Comparison of Promega's luciferase assay system to the conventional luciferase assay method. The sample is an extract made of NIH 3T3 cells expressing the luciferase gene from the Rous sarcoma virus promoter (pRSVL). After 48 hours, the cells were lysed using cell culture lysis reagent (pg. 296). In measurement techniques where the luciferase is mixed with substrates prior to light detection (e.g., scintillation counting), the shaded area represents the light typically lost from measurement. In the conventional assay, this is 50% of the total luminescence in a 1 minute measurement.

*Patent Pending

III. Luciferase Assay System

(continued)

B. Luciferase Assay Protocol

Reconstitute the luciferase assay reagent by adding 10ml of luciferase assay buffer (supplied) to the vial containing lyophilized luciferase assay substrates. Dispense this reagent into aliquots and store at –70°C to avoid repeated thaws. A typical assay requires 100µl of luciferase assay reagent. The assay reagent is stable for 1 month when stored at –20°C or 1 year when stored at –70°C.

Perform all steps with the reagents at room temperature, the optimum temperature for firefly luciferase activity. Consistent temperature is especially important during the actual measurement of light, since the rate of photon production (i.e., light intensity) is temperature sensitive. Under most conditions, firefly luciferase is quite stable at room temperature, losing less than 5% activity in 10 hours. If the cell extracts must be stored between lysis and measurement, store them at –70°C.

Before performing experiments, it is recommended that a dilution series of luciferase be assayed to determine the linear range of the light detection method. This is especially true when using scintillation counters or photon-counting luminometers, since these instruments usually experience signal saturation at high light intensities. Luciferase (purified or from cell culture) should be serially diluted in 1X cell culture lysis reagent containing 1mg/ml BSA (see Note 1). Light intensity is proportional to luciferase concentration in the range of 10^{-16} to 10^{-8}M. For operation of luminometers or scintillation counters, consult the appropriate operator's manual.

The following procedure is optimized for mammalian cells grown in culture. Modifications may be necessary for optimum results with certain cell types (see Note 2).

Reagents Needed

- PBS buffer (pg. 296)
- luciferase assay reagent (supplied, pg. 296)
- cell culture lysis reagent (supplied, pg. 296)

Protocol

1. Dilute the required volume of cell culture lysis reagent to 1X. Allow both the diluted lysis reagent and an aliquot of luciferase assay reagent to come to room temperature (see Note 3).

2. Remove the growth media from the cells to be assayed. Rinse the cells twice in PBS buffer.

3. Add a minimal volume of 1X cell culture lysis reagent to the cells (e.g., 250µl for a 60mm culture dish) and incubate at room temperature for 10-15 minutes.

4. Scrape the attached cells from the culture dish and transfer the cells and solution to a microcentrifuge tube. Spin briefly (about 5 seconds) in a microcentrifuge to pellet large debris.

5. Mix 20µl of the cell extract with 100µl of luciferase assay reagent at room temperature (see Notes 4 and 5) and place the reaction in a luminometer or scintillation counter (see Notes 6 and 7).

6. Measure light produced for a period of 10 seconds to 5 minutes, depending on the sensitivity needed. The light intensity of the reaction is nearly constant for about 20 seconds and then decays slowly with a half-life of about 5 minutes. Thus, it is best to begin measurement within 10 seconds of mixing the extract and assay reagents.

Notes:

1. If dilutions of the sample are required for any reason (such as too much light intensity in the sample for a photon-counting luminometer or scintillation counter), the diluent should be 1X cell culture lysis reagent containing 1mg/ml BSA. The addition of BSA is necessary to ensure that luciferase is not lost from solution by adsorption onto container surfaces.

2. For plant tissue, quick freeze the tissue in liquid nitrogen, grind the frozen tissue to a powder, and resuspend it in room temperature 1X cell culture lysis reagent with further homogenization. For bacteria, pellet the cells, resuspend in cold 1X cell culture lysis reagent and sonicate on ice one or more times for 15-30 seconds each. (The solution is kept cold to compensate for heat generated by the sonication probe.)

(continued on next page)

III. Luciferase Assay System

(continued)

If excessive foaming occurs during sonication, the user snould instead resuspend the cells in a small volume of 50mM Tris-phosphate, pH 7.8 (Tris•H_3PO_4), and sonicate as described above. After sonication, add an equal volume of room temperature 1X cell culture lysis reagent. (This will provide a sufficient concentration of lysis reagents.) For both plant and bacterial cells, remove the debris by centrifugation after cell lysis and then assay the supernatant using standard conditions.

3. Light intensity is a measure of the rate of catalysis by luciferase, and is therefore dependent upon temperature. The temperature optimum for luciferase activity is approximately room temperature (20-25°C). It is important that the luciferase assay reagent be fully equilibrated to room temperature before beginning measurements. One method to help ensure this is to place a thawed aliquot of the assay reagent, in a sealed tube, into a container of water that has been left at room temperature. Leave this to thermally equilibrate for at least 30 minutes.

   The sample to be assayed should also be at room temperature. Generally, luciferase activity will be stable for several hours at room temperature in 1X cell culture lysis reagent. If specific circumstances make this unacceptable, the sample may be left on ice. Assay of a cold sample, using standard assay volumes, will result in a 5 to 10% decrease in enzyme activity.

4. The optimized lysis and luciferase extraction conditions employed by this system are moderately inhibitory to enzyme activity. The volumes recommended in the standard assay (20µl sample + 100µl assay reagent) inhibit luciferase activity approximately 10%. If the sample contains substantial luciferase activity, the volume of the sample in the assay may be reduced to 10µl or less. This will reduce the inhibitory effect of the lysis reagent.

5. The total reaction volume of 120µl is convenient for most light measurement devices. For some methods, such as when using film for activity detection, a larger volume may be desirable. Also, many scintillation counters and luminometers may operate well when using a smaller volume. Changes in the total volume of the assay should not affect the properties of the assay if the relative volumes of sample and assay reagent are maintained.

6. In many luminometers, the photomultiplier tube requires 1-2 seconds to stabilize after a sample is introduced. Therefore, it is often better to begin measurement of luminescence after a delay of a couple of seconds.

7. Scintillation counters should be used in the manual mode because light generated by the luminescent reaction decays slowly ($t_{1/2}$ = approximately 5 minutes). Samples should be introduced into the counting chamber of the instrument shortly after light production is initiated.

   Ideally, the coincidence circuit of the scintillation counter should be turned off. Usually, this is achieved through an option of the programming menu or by a switch within the instrument. If the circuit cannot be turned off, a linear relationship between luciferase concentration and cpm can still be produced by calculating the square root of measured cpm minus background cpm (i.e., $[\text{sample–background}]^{1/2}$).

Composition of Solutions

PBS buffer:

137mM	NaCl
2.7mM	KCl
4.3mM	Na_2HPO_4
1.4mM	KH_2PO_4

The final pH should be 7.1.

Reconstituted luciferase assay reagent:

20mM	Tricine
1.07mM	$(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$
2.67mM	$MgSO_4$
0.1mM	EDTA
33.3mM	DTT
270µM	coenzyme A
470µM	luciferin
530µM	ATP

Cell culture lysis reagent (1X solution):

25mM	Tris-phosphate, pH 7.8
2mM	DTT
2mM	1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid
10%	glycerol
1%	Triton X-100

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

A. General Considerations

The ability to introduce DNA into cultured cells has provided a powerful means for studying the function and control of mammalian genes. Several methods have been developed for introducing DNA into tissue culture cells, and the two most widely used techniques today are calcium phosphate and DEAE-Dextran mediated transfection. Both of these methods appear to facilitate DNA binding to cell membranes and entry of DNA into the cell via endocytosis. Calcium phosphate transfection is the method of choice when the goal is to produce long-term stable transfectants. This method works well for transient expression of transfected genes and also appears to add extra protection against intracellular and serum nucleases (37).

DEAE-Dextran transfection is another efficient, quick method for introducing DNA into many cell types, including some cell suspensions. However, its suitability is limited to transient expression studies and it is not recommended for the production of stable transfectants (38). For transient expression studies using a particular cell type, both protocols should be tried in order to determine the most efficient method for transfection. Table 1 lists recommended transfection protocols for three commonly used cell lines.

1. Calcium Phosphate Mediated Transfection

Calcium phosphate mediated transfection involves mixing DNA directly with $CaCl_2$ and a phosphate buffer to form a fine precipitate which is presented to the cultured cells. This method was first reported by Graham and van der Eb (39). In their study, adenovirus DNA infectivity was enhanced in cultured human KB cells when DNA was coprecipitated with calcium phosphate. This technique for DNA transfer was extended by Wigler (40) by demonstrating that the thymidine kinase gene could be transfected and expressed in mouse cells on a long-term basis. The basis for long-term stability of transferred DNA can be integration of the transfected DNA into the host cell genome (41-43) or autonomous replication of transfected DNA in mini-chromosomal structures (44-46).

The calcium phosphate transfection protocol is routinely used for transfecting many cell types for either transient expression or long-term transfection studies. The protocol is versatile and, although used primarily on adherent cells in culture, can also be modified to transfect cells growing in suspension (47).

Table 1. Comparison of Transfection Protocols Recommended for the CHO, COS and HeLa Cell Lines.

Cell Line	Calcium Phosphate (CaP) Protocol	DEAE-Dextran Protocol
Hamster CHO	*CaP for 16hr, then glycerol shock	Standard DEAE-Dextran with chloroquine for 2.5hr, then DMSO shock
Monkey COS	CaP for 48hr, (no shock)	*DEAE-Dextran pre-treatment with chloroquine, 48hr
Human HeLa	*CaP for 48hr, (no shock)	DEAE-Dextran pre-treatment with chloroquine, 48hr

*The asterisk indicates the transfection protocol that worked better. Comparisons were made between the calcium phosphate protocol (cells exposed to CaP for 4, 16 or 48 hours with and without glycerol shock), DEAE-Dextran pre-treatment protocol (with and without chloroquine for 2.5, 5 or 48 hours) and the standard DEAE-Dextran protocol (with and without chloroquine, 2.5 hours with DMSO shock). Cells were transfected with various reporter plasmids including pSV-β-Galactosidase. The efficiency of transfection was monitored by harvesting cells 48 hours post-transfection and assaying for reporter enzyme activity.

Eukaryotic Gene Regulation

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

2. DEAE-Dextran Mediated Transfection

Another efficient technique for introducing DNA into cultured cells utilizes DEAE-Dextran. McCutchan and Pagano (48) first described details of successful gene transfer using DEAE-Dextran, SV40 DNA and MK cells more than 20 years ago. In the traditional protocols, DNA and DEAE-Dextran are mixed together and applied to the cells. An alternative procedure is to expose the cells initially to DEAE-Dextran, wash the cells and then add DNA (49). This procedure works well with HeLa, KB (49), COS and NIH 3T3 cells (unpublished results), and exposes the cells to DEAE-Dextran, which is cytotoxic, for only a brief period of time.

In general, DEAE-Dextran mediated transfection is successful for transient but not stable transfection of cells (38). As DEAE-Dextran is toxic to cells, transfection conditions for individual cell lines may require careful optimization for both DEAE-Dextran concentration and exposure times; at higher DEAE-Dextran concentrations, the exposure time to cells can be shortened in order to minimize cell death. This protocol may be inappropriate for certain cell lines for which DEAE-Dextran is highly toxic.

3. Factors that Affect Efficiency of Gene Transfer

Transfection efficiencies can be increased in many cell types by additional treatments after the primary exposure of the cells to calcium phosphate-DNA or DEAE-Dextran and DNA. The most effective and routinely used agents are glycerol (50,51), dimethyl sulfoxide (DMSO) (51-53), chloroquine (54) and sodium butyrate (55). Each of these chemicals is toxic to cells. Thus, conditions for transfection of individual cell types must be carefully optimized for reagent concentration and exposure time.

Glycerol and DMSO solutions are applied to cells after DNA has been in association with the cells. The glycerol or DMSO solution (usually 10-20%) is applied to the cells for a period of 30 seconds to a few minutes, and then removed from the cells. The exact mechanism of action is unknown, but these treatments may modify cell membrane structure to enhance uptake of DNA.

Chloroquine is generally applied to the cells simultaneously with DNA, and appears to enhance transfection by binding to DNA (56) and inhibiting intracellular DNA degradation by lysosomes (54).

Sodium butyrate treatment following transfection has been reported to result in higher levels of transient expression (55). This effect may be mediated by a more "active" chromatin structure of newly transfected genes (46). For any of these additional treatments, the optimal concentration and times must be empirically and systematically determined.

A number of factors, in addition to those already addressed, will also affect the successful outcome of gene transfer experiments. For example, certain cell types or cell lines are intrinsically easier to transfect than others, although the exact reason for these differences is currently unknown. Even clonal variability in ability to take up DNA has been reported in mouse L cells (57). Secondly, the cells used for gene transfer must be in prime condition, growing exponentially and "happily" at the time of transfection. Another contributing factor to the success or failure of transfection is the quality of the transferred DNA. Finally, all chemicals and reagents used for cell culture should be of the highest possible quality.

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

4. Transient Expression Studies

Cells are typically harvested 48 to 72 hours post-transfection for studies designed to analyze transient expression of the transferred genes. The optimal time interval depends upon the cell type, the doubling time of the cells and the specific characteristics of expression for the transferred gene. Analysis of gene products may require isolation of RNA or protein for enzymatic activity assays or immunoassays. The method used for cell harvest will depend upon the end-product being assayed. Procedures for isolation of RNA from cultured cells are described on pgs. 125-130.

5. Long-term Transfection Studies

The goal of stable, long-term transfection is to isolate and propagate individual clones containing transferred DNA. Therefore it is necessary to distinguish and select for those cells which have taken up the exogenous DNA from the bulk of non-transfected cells. This screening can be accomplished by drug selection when an appropriate drug resistance marker is included in the transfected DNA. Alternatively, morphological transformation can be used as a selectable trait in certain cases. For example, bovine papilloma virus vectors produce a morphological change in transfected mouse Cl127 cells (58).

Typically, cells are maintained in non-selective medium for 1 to 2 days post-transfection, then trypsinized and replated in selective medium containing the drug. The use of the selective medium is continued for 2 to 3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies are then trypsinized and transferred to microtiter wells for further propagation in the presence of selective medium.

Several different drug selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for aminoglycoside phosphotransferase can be selected for stable transformation in the presence of the drug G418 (59). Similarly, expression of the gene for hygromycin B phosphotransferase from the transfected vector will confer resistance to the drug hygromycin B (60).

An alternative strategy is to use a vector carrying an essential gene that is defective in a given cell line. For example, CHO cells deficient in expression of the dihydrofolate reductase (DHFR) gene survive only in the presence of added nucleosides. However, these cells, when stably transfected with DNA expressing the DHFR gene, will synthesize the required nucleosides (61). An additional advantage of using DHFR as a marker is that gene amplification of DHFR and associated transfected DNA occurs when cells are exposed to increasing doses of methotrexate (62).

Eukaryotic Gene Regulation

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

B. Preparation of Cells and DNA

Many excellent books (63-65) are available which review tissue culture techniques.

1. Trypsinization Procedure for Removing Adherent Cells

Reagents to be Supplied by the User

- calcium and magnesium free salt solution [PBS (pg. 305) or Hank's balanced salt solution]
- trypsin-EDTA solution (pg. 305)

Protocol

Trypsinizing cells for purposes of subculturing or cell counting is an important technique which is critical to successful cell culture. The following technique works consistently well when passaging cells.

a. Prepare a sterile trypsin-EDTA solution (pg. 305) in a calcium and magnesium free salt solution, pH 7.4 to 7.6 (e.g., Hank's balanced salt solution or PBS). The 1X solution can be frozen and thawed for future use, but the activity of the trypsin will decline.

b. Remove the media from the tissue culture dish and wash the cells twice with the calcium and magnesium free salt solution. At this stage it is important to remove residual serum which would inactivate trypsin.

c. After removing the last wash, add just enough of the trypsin solution to cover the cell monolayer: 2ml for a 150mm flask, 1ml for a 100mm plate. Rock the plates to distribute the trypsin solution evenly. Place the plates in a 37°C incubator for 1 to 2 minutes, just until the cells begin to detach. The length of the trypsin incubation required depends on the cell type and must be monitored closely because excess exposure of cells to trypsin is toxic. The following incubation times for a few cell lines are provided as guidelines.

Cell Line	Trypsin Incubation Time
CHO	1 minute
COS	2 minutes
HeLa	1.5 minutes
NIH 3T3	1 minute

d. Once the cells begin to detach, QUICKLY REMOVE the trypsin solution (tilt the plate to get off as much as possible with a pipet). Strike the bottom and sides of the culture vessel sharply with the palm of your hand to dislodge the remaining adherent cells.

e. Add media containing a minimum of 7% serum to the cells to inactivate the trypsin. Gently pipette the cells up and down to break up cell clumps. The cells may now be counted and/or distributed to fresh plates for subculturing.

2. Plating Cells for Transfection

Plate cells the day before the transfection experiment. The plating density for any particular cell line will depend on how quickly the cells divide. The cells should be at 30-60% confluency the day of the transfection. An optimal plating density produces a nearly confluent dish when the cells are harvested or split into selective media, which is usually about 48 hours after the transfection. A general guideline is to plate about 8×10^5 cells per 100mm culture dish. Scale down the number of cells proportionately for 35 or 60mm dishes.

3. Preparation of DNA for Transfection

Supercoiled plasmid DNA is efficiently expressed following both calcium phosphate and DEAE-Dextran mediated transfections. The plasmid DNA to be used in the transfection should be free of protein, RNA and chemical contamination. Ethanol-precipitated DNA should be resuspended in a sterile solution such as TE buffer (pg. 305) to a final concentration of 0.2-1mg/ml. A good indicator of DNA purity is the ratio of optical densities (OD) at 260 versus 280nm. A DNA solution with an O.D. 260/280 ratio of 1.8 or greater is desirable. For a detailed protocol for plasmid DNA purification, see pgs. 106-107.

Promega

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

C. Calcium Phosphate Transfection

Reagents to be Supplied by the User

- growth medium

Protocol

1. Calcium Phosphate Transfection Protocol

a. Plate the cells the day before the transfection experiment as described in Section B.2, above.

b. Three hours prior to the transfection, remove the medium from the cells and replace it with fresh growth medium.

c. Thaw all system components and warm them to room temperature. Mix each component thoroughly by swirling the container or vortexing.

d. For each transfection, prepare the DNA and HBS 2X buffer solutions in separate sterile tubes:

	per 60mm dish	per 100mm dish
DNA	6-12µg	10-20µg
2M $CaCl_2$	37µl	62µl
sterile, deionized water	to 0.3ml final	to 0.5ml final
HBS 2X buffer (pg. 305)	0.3ml	0.5ml

e. Vortex the HBS 2X buffer, adjusting the speed such that the tube can be vortexed safely with the cap off and can accommodate the addition of the prepared DNA solution. Continue to vortex while slowly adding the prepared DNA solution dropwise to the HBS. (Alternatively, bubble air with a pipette through the HBS while slowly adding the $CaCl_2$/DNA solution.) When the DNA addition is complete, the solution should appear slightly opaque due to formation of a fine co-precipitate of DNA with calcium and phosphate. Incubate the solution at room temperature for 30 minutes.

f. Vortex the transfection solution again just prior to adding it to cells. Add the solution dropwise to plates (0.6ml per 60mm plate, 1ml per 100mm plate). Swirl the plates to mix. Return the plates to a 37°C CO_2 incubator. OPTIONAL: A glycerol or DMSO shock step may be performed 4 to 16 hours after addition of the DNA to the cells (refer to Section E, pg. 304).

g. In general, cells may be harvested or selective media applied 48-72 hours after the transfection.

2. Optimizing the Calcium Phosphate Protocol

a. Optimally, the calcium phosphate-DNA coprecipitate should be fine in appearance. Several factors influence the formation of this precipitate.

- The $CaCl_2$/DNA and HBS 2X buffer which are mixed should be at room temperature (22-25°C). Higher or lower temperatures for precipitate formation can lead to decreased transfection efficiency.
- The addition of $CaCl_2$/DNA to the HBS 2X buffer should be performed dropwise and with continuous mixing.
- The concentration of DNA can affect the size of the precipitate. Low amounts of DNA (less than 1µg) can be supplemented with carrier DNA such as salmon or herring sperm DNA. However, there are conflicting reports in the literature as to the efficacy of adding carrier DNA (52,66).
- High molecular weight DNA can affect the size of the precipitate. A more uniform precipitate forms if the DNA is first sheared before mixing with $CaCl_2$.

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

b. Efficient transfection occurs in a very narrow range of pH.

- A large volume of added DNA in Tris buffer could change the pH. Therefore, the DNA used for transfection should be resuspended in water, 1mM Tris or, if present in 10mM Tris, should be fairly concentrated so that a relatively small volume of the DNA solution is added to the HBS.
- The precipitate should be added dropwise around the dish to the medium bathing the cells, and the medium should be mixed thoroughly at the end of the addition. This helps to evenly distribute the precipitate and avoid localized acidification of cells.
- After addition of the precipitate to the cells, the pH of the medium should be between 7.2 and 7.4, and CO_2 should be maintained at 5%.

D. DEAE-Dextran Transfection

Two different protocols are listed below. The first protocol involves pretreatment of the cells with DEAE-Dextran and is a modification of the procedure described by Al-Molish, *et al.* (49). It offers the advantage of a limited DEAE-Dextran exposure (long exposures of DEAE-Dextran may be toxic to some cell lines), followed by a longer DNA incubation, allowing maximal DNA uptake. The second protocol is a more standard protocol which includes a concurrent exposure of cells to DEAE-Dextran and DNA. The best protocol for a particular cell line should be determined experimentally. The addition of 80µM chloroquine along with the DNA is an option for both protocols. Chloroquine appears to act by decreasing degradation of the transfecting DNA (54). For some cell lines, chloroquine dramatically increases transfection efficiencies while for others, it has a minimal effect and may be quite cytotoxic.

Both protocols require a sterile, calcium- and magnesium-free salt solution for the wash steps. This wash solution is not provided with the system. PBS (pg. 305) or another salt solution such as Hank's balanced salt solution works well for this purpose.

Reagents to be Supplied by the User

- calcium- and magnesium-free salt solution [PBS (pg. 305) or Hank's balanced salt solution]
- growth medium
- chloroquine (optional)

1. DEAE-Dextran Pretreatment Protocol

a. Plate the cells the day before the transfection experiment as described in Section B.2, pg. 300.

b. Prepare the wash solution (not supplied) and warm to 37°C. For each 60mm plate, you will need 15ml of sterile PBS (pg. 305) or Hank's balanced salt solution for the washes. For 100mm plates, you will need 30ml per plate.

c. Prepare the transfection solutions:

i. Dilute the PBS 10X stock (supplied) 10-fold with distilled or deionized water and sterilize it by filtration. You will need approximately 3ml of PBS per 60mm plate or 5ml per 100mm plate.

ii. Dilute the DEAE-Dextran stock solution 1:10 in the PBS solution prepared above. You will need 2ml of diluted DEAE-Dextran per 60mm plate or 4ml per 100mm plate.

iii. Dilute the DNA in PBS to a final volume of 325µl for a 60mm plate or 540µl for a 100mm plate.

d. Remove the medium from the cells. Add sterile wash solution (PBS or Hank's balanced salt solution): 5ml for 60mm plates, 10ml for 100mm plates. Incubate for 15 minutes at room temperature.

e. Remove the wash solution from the cells. Add diluted DEAE-Dextran solution: 2ml per 60mm plate, 4ml per 100mm plate. Incubate for 9 minutes at room temperature.

f. Remove the DEAE-Dextran solution. Very gently wash the cells twice with PBS or Hank's salt solution (2 x 5ml per 60mm plate, 2 x 10ml per 100mm plate). Aspirate the final wash.

g. Add the diluted DNA to the cells (325µl per 60mm plate, 540µl per 100mm plate). Dispense evenly over the cells. Incubate for 30 minutes in a 37°C CO_2 incubator. Rock the plates occasionally to keep the cells moist.

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IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

h. Add 3.5ml (per 60mm plate) or 6ml (per 100mm plate) of regular growth medium.

OPTIONAL: Add 8mM chloroquine (35µl per 60mm plate, 60µl per 100mm plate) together with the medium.

i. Return the plates to a 37°C CO_2 incubator.

If the cells are sensitive to chloroquine (determined empirically), change the medium on the plates before cytotoxic effects occur (often between 2-5 hours). With hardy cells such as HeLa or NIH 3T3, the chloroquine/DNA mixture can be left on the cells up to 48 hours.

j. In general, cells may be harvested 48-72 hours after the transfection.

2. Standard DEAE-Dextran Protocol

a. Plate the cells the day before the transfection experiment as described in Section IV.B, pg. 300.

b. Prepare the wash solution (not supplied) and warm it to 37°C. For the washes, 10ml of PBS (pg. 305) or calcium and magnesium free Hank's balanced salt solution are required for each 60mm plate and 20ml for each 100mm plate. Also warm the DEAE-Dextran solution to 37°C.

c. Prepare the transfection solutions:

i. Dilute the PBS 10X stock (supplied) 10-fold with distilled or deionized water and sterilize it by filtration. You will need approximately 0.4ml of PBS per 60mm plate or 0.6ml per 100mm plate.

ii. Dilute the DNA in PBS and add DEAE-Dextran.

Per 60mm dish: Dilute the DNA to a final volume of 326µl in PBS. Add 17µl of 10mg/ml DEAE-Dextran.

Per 100mm dish: Dilute the DNA to a final volume of 540µl in PBS. Add 28µl of 10mg/ml DEAE-Dextran.

d. Remove the medium from the cells. Wash the cells twice with calcium- and magnesium-free wash solution (2 x 5ml per 60mm plate, 2 x 10ml per 100mm plate).

e. Add the DNA/DEAE-Dextran mixture: 343µl per 60mm plate, 568µl per 100mm plate. Dispense evenly over the cells. The concentration of DEAE-Dextran exposed to the cells is 0.5mg/ml.

f. Incubate for 30 minutes at 37°C. Rock the plates occasionally to keep the cells moist.

g. Gently add 3.5ml of growth medium per 60mm plate or 6ml of medium per 100mm plate. Incubate up to 2.5 hours at 37°C, or until cytotoxicity is apparent. Then gently change the medium or follow with a DMSO step (see Section E.2, pg. 304).

OPTIONAL: Add 8mM chloroquine (35µl per 60mm plate, 60µl per 100mm plate) along with the medium during the 2.5 hour incubation step.

h. In general, cells may be harvested 48-72 hours after the transfection.

3. Optimizing the DEAE-Dextran Protocol

a. Because of the various degrees of toxicity of DEAE-Dextran and chloroquine to different types of cells, it is important to watch for signs of cell death and toxicity. If toxicity is a problem, then the concentration of DEAE-Dextran or the exposure time of DEAE-Dextran and chloroquine to the cells can be decreased.

b. Certain types of cells that are very sensitive to DEAE-Dextran toxicity, such as primary cell cultures, may require a higher cell concentration at the time of transfection.

c. For some cell lines, lower concentrations of DNA can be used for standard DEAE-Dextran transfections compared to calcium phosphate transfections. A dose-response curve could be established to determine the optimal DNA concentration to use.

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

E. Glycerol or DMSO Shocks

1. Glycerol Shock

The glycerol shock is generally applied to cells exposed to calcium phosphate. For some cell lines, a glycerol shock step is unnecessary and does not increase the efficiency of transfection. High transfection efficiencies can be obtained by leaving the DNA/calcium phosphate solution on the cells until the cells are harvested or selective pressure is applied. HeLa cells, for example, respond well to this treatment. However, transfection of some cell lines, such as hamster CHO cells, is enhanced by the glycerol shock step (see Table 1, pg. 297). For this reason, we have included a representative glycerol shock protocol below.

The glycerol shock step may be performed 4-16 hours after transfection. In general, if cells can tolerate the calcium phosphate solution, it is best to leave it on for as long as possible and perform the glycerol shock 16 hours after transfection. Some cell lines may be more sensitive to the calcium phosphate solution and may respond better to a glycerol shock step performed earlier, such as 4 hours after exposure to the DNA. The optimum time interval before performing the glycerol shock should be determined empirically for each cell line.

Reagents to be Supplied by the User

- calcium- and magnesium-free salt solution [PBS (pg. 305) or Hank's balanced salt solution]
- glycerol
- growth medium

Protocol

a. Prepare a fresh 15% glycerol shock solution in HBS buffer (pg. 305) and warm it to 37°C, along with regular growth media and a balanced salt solution such as PBS (pg. 305) or Hanks balanced salt solution.

b. Wash the cells once with the balanced salt solution.

c. Add the glycerol shock solution: 2ml per 60mm plate, 3ml per 100mm plate.

d. Incubate for 2 minutes at room temperature.

e. Remove the glycerol solution and wash the cells twice with the balanced salt solution.

f. Add regular growth medium and return the cells to a 37°C incubator.

2. DMSO Shock

Certain cell types exhibit enhanced transfection efficiencies after exposure to dimethyl sulfoxide (DMSO). The DMSO step can be added to either the calcium phosphate or DEAE-Dextran transfection protocols (51-53). DMSO, like glycerol, is toxic to cells and the concentration and exposure times may require careful optimization for each cell type. One representative protocol for a DMSO shock is provided below.

Reagents to be Supplied by the User

- calcium- and magnesium-free salt solution [PBS (pg. 305) or Hank's balanced salt solution]
- DMSO (tissue culture grade)

Protocol

a. Prewarm the PBS to 37°C and add DMSO to a final concentration of 10% immediately before use (2ml per 60mm plate, 3ml per 100mm plate).

b. Remove the medium from the cells.

c. Add 10% DMSO in PBS to the cells and incubate for 2.5 minutes.

d. Remove the DMSO and add regular growth medium (5ml per 60mm plate, 10ml per 100mm plate). Return the cells to the 37°C incubator.

IV. Transfection of Eukaryotic Cells with the ProFection™ Systems

(continued)

Composition of Buffers and Solutions

HBS (HEPES-buffered saline) 2X buffer:

50mM	HEPES, pH 7.1 [HEPES: 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid]
280mM	NaCl
1.5mM	Na_2HPO_4

PBS buffer:

137mM	NaCl
2.7mM	KCl
4.3mM	Na_2HPO_4
1.47mM	KH_2PO_4

The final pH should be 7.3.

Trypsin-EDTA 1X solution:

0.05% (w/v)	trypsin
0.53mM	EDTA

Dissolve these components in a calcium- and magnesium-free salt solution, pH 7.4 to 7.6, such as PBS or Hank's balanced salt solution.

TE buffer:

10mM	Tris-HCl, pH 8.0
1mM	EDTA

Glycerol shock solution:

15%	glycerol in HBS 1X buffer

DMSO shock solution:

10%	DMSO (tissue culture grade) in PBS buffer

TEN buffer:

40mM	Tris-HCl, pH 7.5
1mM	EDTA
150mM	NaCl

Eukaryotic Gene Regulation

V. Affinity Purification of DNA Binding Proteins with the GRAB System

This system is designed for the study of sequence-specific DNA binding proteins. Based on the method developed by Levens and Howley (67), putative control regions can be used as affinity probes to identify binding factors. Binding sites on DNA also can be mapped using this system (see Figure 6, pg. 307). The above reference should be used as a guideline for these applications.

For most applications, the anti-β-galactosidase monoclonal antibody is first coupled to a solid support, such as Immunobeads (Bio-Rad #170-5910). This matrix is suitable because of its good mechanical properties and low non-specific binding characteristics. Covalent attachment of the antibody to the polyacrylamide beads is via peptide bond formation using EDAC, a water-soluble carbodiimide. Since the antibody is supplied in a Tris buffer (for long term stability), it must be transferred to 3mM phosphate buffer, pH 6.3, prior to coupling. This can be easily accomplished by dialysis, but should be done immediately before coupling since the antibody tends to precipitate with time in this buffer. The coupling protocol supplied by the manufacturer should be followed carefully.

A. Positive Control Assay for Receptor Binding

This control system includes a *lac* operator-containing plasmid to be used as a positive control to verify (if desired) the repressor activity of the *lac*I/Z fusion protein (supplied). The method is a simple filter binding assay based on the specific retention of the *lac*I/Z repressor complexed with operator-containing DNA on nitrocellulose membranes (68,69). The DNA, which can be labeled by a variety of methods, is incubated with increasing amounts of the fusion protein and filtered through nitrocellulose disks. (If the DNA is labeled to a reasonably high specific activity, such as by a brief nick translation, a small amount of labeled DNA can be diluted with a known amount of unlabeled DNA as described below.) As operator sites on the target DNA become saturated, the proportion of the total radioactivity specifically retained on the filters reaches a plateau. The amount of *lac*I/Z fusion protein required to retain 50% of this plateau value is a measure of repressor activity.

B. Labeling of Plasmid DNA

Reagents Required

- nick translation system (Cat.# U1001)
- 1μg positive control plasmid DNA (pGEM®-3Zf(–) supercoiled DNA)
- 1μg negative control plasmid DNA (not containing *lac*Z operator, such as pGEM-3 vector or pBR322 supercoiled DNA)
- 100μCi [^{32}P]dCTP (400Ci/mmole)
- two 2ml (bed volume) disposable columns of Sephacryl S-300, Sepharose 4B, or similar resin
- nick translation 10X buffer (pg. 308)
- enzyme mix (pg. 308)
- stop solution (pg. 308)

A convenient way to prepare labeled DNA suitable for the filter binding assay is by a brief nick translation reaction. Whatever method of labeling is used, it is important to purify the labeled DNA afterward by gel filtration. This serves to remove small degradation products, unincorporated nucleotides, and inhibitors which interfere with the binding assay.

Protocol

1. Both the positive and negative control plasmid DNAs need to be labeled. Assemble a standard 50μl nick translation reaction using 50μCi of label for each plasmid.

Standard Reaction

deionized water	23μl
nucleotide mix (0.1mM dATP, dTTP and dGTP)	10μl
nick translation 10X buffer (pg. 308)	5μl
DNA (plasmid stock 0.5μg/μl)	2μl
[α-^{32}P]dCTP (400Ci/mmole) at 10mCi/ml	5μl
optimized enzyme mix	5μl
	total volume 50μl

2. Incubate at 15°C for 15 minutes. Stop the reaction by adding 5μl of stop solution. Under these conditions, the DNA becomes sufficiently labeled for filter binding assays, but at least 90% remains intact as full-length linear molecules on native agarose gels. This is, of course, important since it is necessary to retain the integrity of the operator sites.

V. Affinity Purification of DNA Binding Proteins with the GRAB System

(continued)

3. Run the reaction over a 2ml gel filtration column (e.g., Sephacryl® S300*, Sepharose 4B) using TE buffer (pg. 305) and collect 6-drop fractions. Use the void fraction(s) directly for the filter binding assay. (*Sephacryl is a registered trademark of Pharmacia, Inc.)

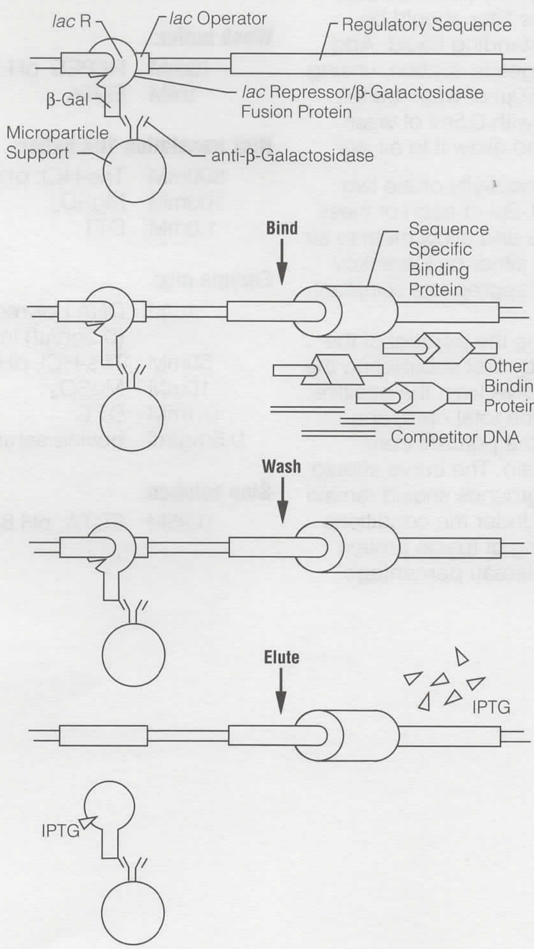

Figure 6. Schematic diagram of the GRAB system procedure.

C. Filter Binding Assay for DNA Binding Proteins

Reagents Required

- ^{32}P-labeled positive and negative control DNAs
- unlabeled, *Eco*R I-digested positive control plasmid DNA
- unlabeled, linearized negative control plasmid DNA
- *lac*I/Z fusion protein (freshly diluted to 10ng/µl in binding buffer)
- binding buffer (pg. 308)
- wash buffer (pg. 308)

Protocol

1. Mix about 100,000cpm of labeled positive control DNA or sample DNA containing the regulatory sequence of interest (usually 20-30µl of the pooled fractions above) with 10µl of unlabeled positive control DNA (50ng/µl = 500ng) and make a similar mixture of labeled and unlabeled negative control DNAs in a separate tube.

2. Assemble 16 numbered microcentrifuge tubes with the following components at room temperature. The amount of DNA mixture will vary; add that amount which corresponds to 50ng of unlabeled DNA. In this example we have used 4µl. Add the fusion protein last since this will start the reaction.

Tube#	DNA Mixture	Binding Buffer	10ng/µl Fusion Protein
1	4µl	46µl	0µl
2	4µl	45µl	1µl
3	4µl	44µl	2µl
4	4µl	41µl	5µl
5	4µl	36µl	10µl
6	4µl	34µl	12µl
7	4µl	31µl	15µl
8	4µl	26µl	20µl

For tubes #1-8 use the positive control DNA mixture. For tubes #9-16 use the non-operator negative control DNA mixture with the same amounts of fusion protein and binding buffer.

Eukaryotic Gene Regulation

V. Affinity Purification of DNA Binding Proteins with the GRAB System

(continued)

3. Incubate the reactions at room temperature for 10 minutes. Since equilibrium is reached during this incubation, the samples can be placed on ice until they are filtered.

4. To filter the reactions, place a nitrocellulose filter disk on a filter housing. Wet the filter with about 0.5ml of wash buffer and gently pull the liquid through under vacuum. The filter should be moist without any excess standing liquid. Add the sample dropwise with gentle suction, rinsing out the reaction tube with 50µl of wash buffer, and rinse the filter 3 times with 0.5ml of wash buffer. Remove the filter and allow it to air dry.

   To determine the total radioactivity of the two DNA mixtures used, spot 1-2µl of each of these mixtures on separate filters and allow them to air dry. Count all of the filters, either by Cerenkov radiation or after adding an appropriate scintillant.

5. For each sample, determine the percent of the DNA specifically retained by first subtracting the cpm for the non-operator DNA from the positive control value, dividing by the total cpm, and multiplying by 100%. Plot the percent cpm retained vs. ng fusion protein. The curve should rise to a plateau and backgrounds should remain low (5-10% of total cpm). Under the conditions of this assay, less than 50ng of fusion protein should retain 50% of the plateau percentage.

Composition of Solutions

Binding buffer:

10mM	HEPES, pH 7.5
1mM	EDTA
5%	glycerol
50µg/ml	bovine serum albumin
1mM	DTT

Wash buffer:

10mM	HEPES, pH 7.5
1mM	EDTA

Nick translation 10X buffer:

500mM	Tris-HCl, pH 7.2
100mM	$MgSO_4$
1.0mM	DTT

Enzyme mix:

1u/µl	DNA polymerase I (1u/µl) and DNase I (0.2ng/µl) in a 50% glycerol solution
50mM	Tris-HCl, pH 7.2
10mM	$MgSO_4$
0.1mM	DTT
0.5mg/ml	bovine serum albumin

Stop solution:

0.25M	EDTA, pH 8.0

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VI. References

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Eukaryotic Gene Regulation

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VII. Additional Eukaryotic Gene Regulation Literature Available from Promega

Manual

ProFection™ Mammalian Transfection Systems Technical Manual

Technical Bulletins

060 Gene Regulator Affinity Binding System: Repressor Activity Assay

060 pCAT®-Basic Vector

081 pCAT®-Control Vector

082 pCAT®-Enhancer Vector

083 pCAT®-Promoter Vector

084 CAT Assay System

094 pSV-β-Galactosidase Control Plasmid

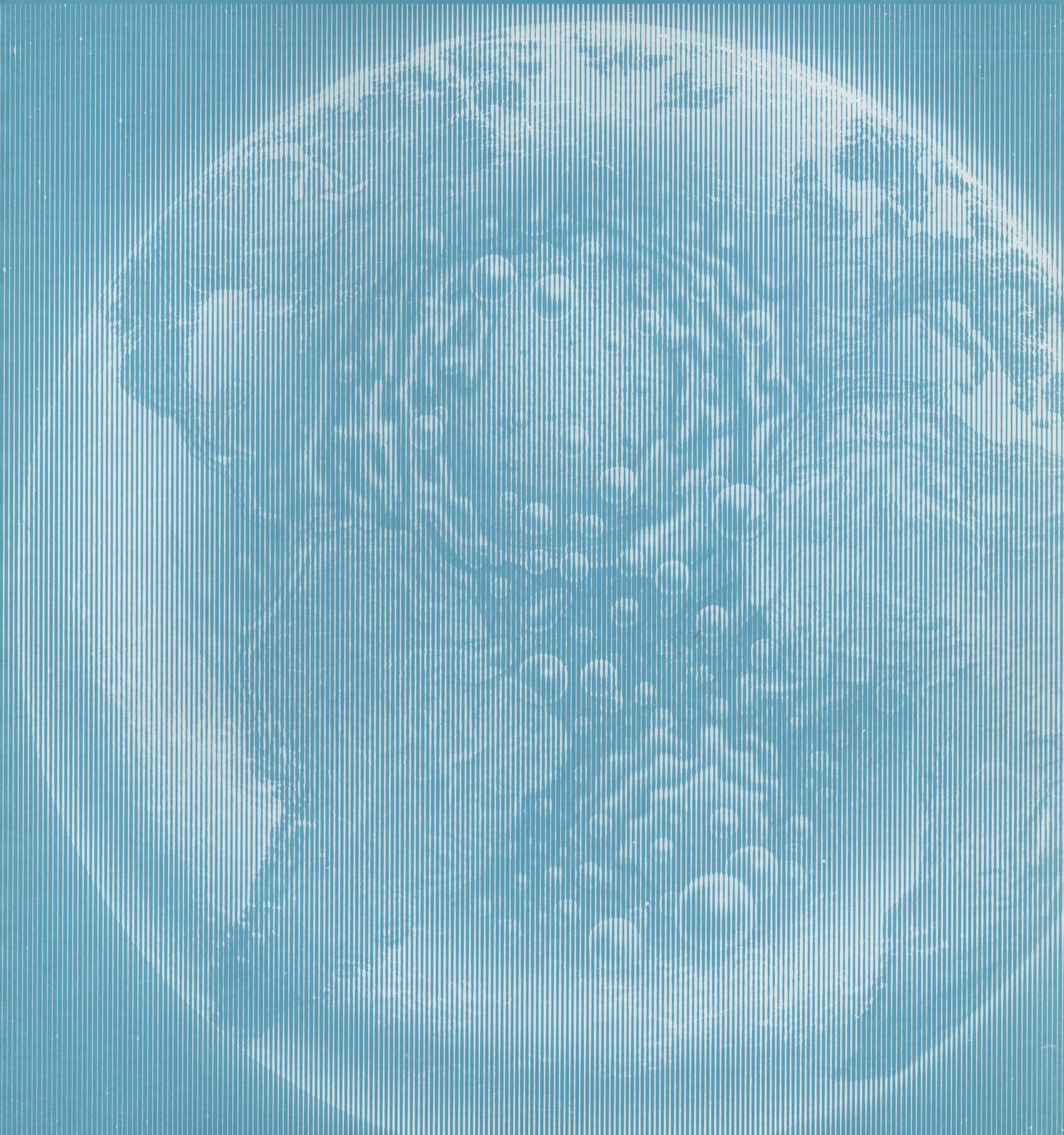
097 β-Galactosidase Assay System

Promega Notes Articles

Issue	Title
21	Detection of eukaryotic transcriptional regulatory sequences with the CAT reporter gene assay
28	Firefly luciferase: a new tool for molecular biologists

Technical Services:
800-356-9526

PROTOCOLS AND APPLICATIONS GUIDE



Vector Maps

Contents

Note: U.S. Pat. No. 4,766,072 has been issued to Promega Corporation for transcription vectors having two different promoter sequences separated by a series of unique restriction sites into which foreign DNA can be inserted.

Vector Maps

Plasmid Properties

Table 1. Features and Applications of Riboprobe, Riboprobe Gemini, pGEMEX and pSELECT Vectors.

	pSP64, pSP65 & pSP64(polyA)	pSP70 & pSP71	pSP72 & pSP73	pGEM-1 & pGEM-2	pGEM-3 & pGEM-4	pGEM-3Z & pGEM-4Z	pGEM-3Zf(+) & pGEM-3Zf(–)	pGEM-5Zf(+) & pGEM-5Zf(–)	pGEM-7Zf(+) & pGEM-7Zf(–)	pGEM-9Zf(–)	pGEM-11Zf(+) & pGEM-11Zf(–)	pGEM-13Zf(+)	pGEMEX-1 & pGEMEX-2	pSELECT-1
Size (bp)	2,999 3,005 3,033	2,417 2,419	2,642 2,464	2,865 2,869	2,867 2,871	2,743 2,746	3,199 3,199	3,003	3,000	2,925	3,223	3,181	3,995 3,997	5,680
Ampicillin selection	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
Promoters	S	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T7	S,T3,T7	S,T7
Blue/white screening for recombinants	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	N	Y
Single-stranded DNA production	N	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y
Direct dsDNA sequencing possible	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Sequencing primers applicable	S	S,T7	S,T7	S,T7	S,T7	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 M	S,T7 T3	S,T7 M
Classification	R	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG	RG

Key: R - Riboprobe   S - SP6
RG - Riboprobe Gemini   M - M13

Note: "pGEM" is a registered trademark of Promega Corporation and should be referred to as pGEM® vector.

Promega

Plasmid Properties

Table 2. Summary of Restriction Sites in Mulitple Cloning Regions of Riboprobe, Riboprobe Gemini, pGEMEX and pSELECT Vectors.

	pSP64, pSP65 & pSP64(polyA)	pSP70 & pSP71	pSP72 & pSP73	pGEM-1 & pGEM-2	pGEM-3 & pGEM-4	pGEM-3Z & pGEM-4Z	pGEM-3Zf(+) & pGEM-3Zf(–)	pGEM-5Zf(+) & pGEM-5Zf(–)	pGEM-7Zf(+) & pGEM-7Zf(–)	pGEM-9Zf(–)	pGEM-11Zf(+) & pGEM-11Zf(–)	pGEM-13Zf(+)	pGEMEX-1 & pGEMEX-2	pSELECT-1	
Aat II								●	●						*Aat* II
Acc I	●		●	●	●	●	●				●		●		*Acc* I
Ava I	●			●	●	●	●								*Ava* I
Apa I								●	●		●		●		*Apa* I
BamH I	●		●	●	●	●	●		●		●		●	●	*BamH* I
Bgl II		●	●												*Bgl* II
BstX I								●	●						*BstX* I
Cla I		●	●						●						*Cla* I
Csp45 I									●						*Csp45* I
Eag I								●			●		●		*Eag* I
EcoR I	●	●	●	●	●	●	●		●	●	●		●	●	*EcoR* I
EcoR V		●	●					●							*EcoR* V
Hind II	●			●	●	●	●				●		●		*Hind* II
Hind III	●	●	●	●	●	●	●		●	●	●	●	●	●	*Hind* III
Kpn I			●		●	●	●		●					●	*Kpn* I
Nco I								●							*Nco* I
Nde I								●							*Nde* I
Not I								●		●	●	●	●		*Not* I
Nsi I								●	●	●	●		●		*Nsi* I
Pst I	●		●	●	●	●	●	●						●	*Pst* I
Pvu II		●	●												*Pvu* II
Sac I	●		●	●	●	●	●	●	●	●	●		●	●	*Sac* I
Sac II								●							*Sac* II
Sal I	●		●	●	●	●	●	●		●	●		●	●	*Sal* I
Sfi I										●	●	●	●		*Sfi* I
Sph I (*Bbu* I)			●		●	●	●	●	●		●		●	●	*Sph* I (*Bbu* I)
Sma I	●		●	●	●	●	●		●					●	*Sma* I
Spe I								●		●					*Spe* I
Tth111 I										●					*Tth111* I
Xba I	●		●	●	●	●	●		●	●	●		●	●	*Xba* I
Xma I									●						*Xma* I
Xho I		●	●						●		●		●		*Xho* I

Note: "pGEM" is a registered trademark of Promega Corporation and should be referred to as pGEM® vector.

Vector Maps

pGEM®-1

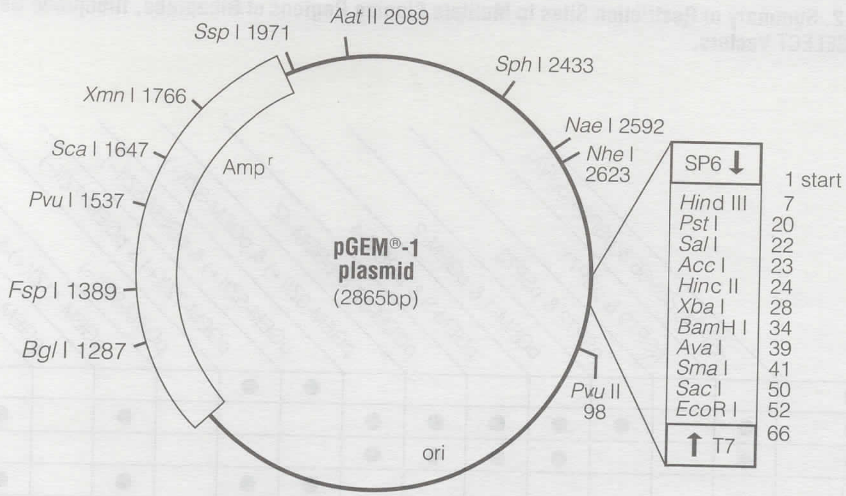


Figure 1. pGEM-1 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 66
   c. SP6 RNA polymerase promoter 2849-2865
   d. T7 RNA polymerase promoter 67-83
   e. multiple cloning sites 7-57
   f. *lac* operon sequences 94-152
   g. β-lactamase (Amp^r) region 1094-1954
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pGEM-1 and -2 vectors are identical except for the orientation of the multiple cloning site region.

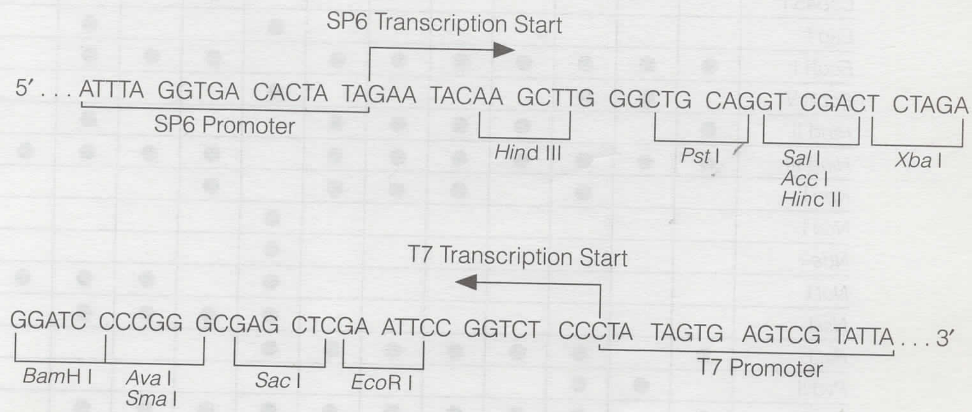


Figure 2. pGEM-1 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Technical Services:
800-356-9526

Table 3. pGEM-1 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2089
Acc I	1	23
Acc III	0	
Acy I	5	1704, 2086, 2444, 2558, 2579
Afl II	0	
Alu I	13	9, 48, 98, 216, 442, 532, 578, 835, 1356, 1456, 1519, 2198, 2217
Alw44 I	3	588, 1834, 2331
Apa I	0	
Asu I	6	1209, 1288, 1305, 1527, 2143, 2468
Asu II	0	
Ava I	1	39
Ava II	2	1305, 1527
Bal I	0	
BamH I	1	34
Ban I	4	1115, 2443, 2557, 2578
Ban II	3	50, 2510, 2524
Ban III	0	
Bbu I	1	2433
Bbv I	12	2, 85, 166, 184, 603, 693, 696, 902, 1205, 1596, 2207, 2389
Bcl I	0	
Bgl I	1	1287
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	50, 592, 1753, 1838, 2335, 2408, 2510, 2524
BspM I	1	9
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	3	302, 423, 436
BstX I	0	
Bsu 36 I	0	
Cfo I	18	123, 151, 184, 454, 521, 621, 795, 904, 1297, 1390, 1727, 2059, 2159, 2262, 2446, 2500, 2560, 2581
Cfr I	4	113, 1555, 2460, 2592
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	7	549, 958, 1124, 1664, 2090, 2325, 2689
Dpn I	15	36, 842, 917, 928, 936, 1014, 1026, 1131, 1472, 1490, 1536, 1794, 1811, 1847, 2527, 2618, 2865
Dra I	3	1033, 1052, 1744
Dra II	2	2143, 2468
Dra III	0	
Eco47 III	1	2499
Eco52 I	0	
EcoR I	1	52
EcoR V	0	
Fnu4H I	22	16, 99, 180, 198, 201, 319, 474, 617, 682, 685, 891, 1219, 1558, 1585, 1680, 1909, 2196, 2305, 2378, 2412, 2415, 2807
Fok I	4	1133, 1314, 1601, 2244
Fsp I	1	1389
Hae II	6	152, 522, 2447, 2501, 2561, 2582
Hae III	13	115, 289, 300, 318, 752, 1210, 1290, 1557, 2144, 2398, 2462, 2470, 2594
Hga I	6	385, 963, 1693, 2251, 2352, 2591
HgiA	6	50, 592, 1753, 1838, 2335, 2408
Hha I	18	123, 151, 184, 454, 521, 621, 795, 904, 1297, 1390, 1727, 2059, 2159, 2262, 2446, 2500, 2560, 2581
Hinc II	1	24
Hind II	1	24
Hind III	1	7
Hinf I	9	25, 73, 109, 174, 249, 645, 1162, 2360, 2714
Hpa I	0	
Hpa II	18	40, 57, 481, 628, 654, 844, 1248, 1282, 1349, 1459, 1701, 2202, 2236, 2459, 2582, 2591, 2606, 2661
Hph I	9	1010, 1237, 1653, 1859, 1894, 2178, 2187, 2551, 2596
Kpn I	0	
Mae I	5	29, 769, 1022, 1357, 2624
Mae III	11	630, 693, 809, 1092, 1423, 1481, 1634, 1822, 2210, 2677, 2854
Mbo I	16	34, 840, 915, 926, 934, 1012, 1024, 1129, 1470, 1488, 1534, 1792, 1809, 1845, 2525, 2616
Mbo II	7	145, 936, 1007, 1762, 1840, 1949, 2520
Mlu I	0	
Mnl I	14	24, 122, 173, 381, 456, 705, 1105, 1186, 1334, 1540, 2133, 2193, 2387, 2606
Msp I	18	40, 57, 481, 628, 654, 844, 1248, 1282, 1349, 1459, 1701, 2202, 2236, 2459, 2582, 2591, 2606, 2661
Mst I	1	1389
Nae I	1	2592
Nar I	3	2444, 2558, 2579
Nci I	9	40, 41, 654, 1350, 1701, 2202, 2237, 2459, 2662
Nco I	0	
Nde I	0	
Nhe I	1	2623
Nla III	11	278, 998, 1489, 1499, 1577, 1613, 2006, 2111, 2195, 2433, 2506
Nla IV	12	36, 306, 345, 1117, 1211, 1252, 1463, 2053, 2445, 2469, 2559, 2580
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	20
Pvu I	1	1537
Pvu II	1	98
Rsa I	3	1647, 2323, 2787
Rsr II	0	
Sac I	1	50
Sac II	0	
Sal I	1	22
Sau3A I	16	34, 840, 915, 926, 934, 1012, 1024, 1129, 1470, 1488, 1534, 1792, 1809, 1845, 2525, 2616
Sau96 I	6	1209, 1288, 1305, 1527, 2143, 2468
Sca I	1	1647
ScrF I	12	40, 41, 302, 423, 436, 654, 1350, 1701, 2202, 2237, 2459, 2662
SfaN I	9	371, 1423, 1614, 1863, 2222, 2316, 2577, 2589, 2756
Sfi I	0	
Sin I	2	1305, 1527
Sma I	1	41
Spe I	0	
Sph I	1	2433
Spo I	0	
Ssp I	1	1971
Sst I	1	50
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	23, 50, 87, 374, 1818, 2341
Tha I	11	121, 123, 321, 902, 1232, 1725, 2057, 2157, 2159, 2262, 2806
Tth111 II	3	864, 871, 903
Xba I	1	28
Xho I	0	
Xho II	8	34, 915, 926, 1012, 1024, 1792, 1809, 2616
Xma I	1	39
Xma III	0	
Xmn I	1	1766

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-2

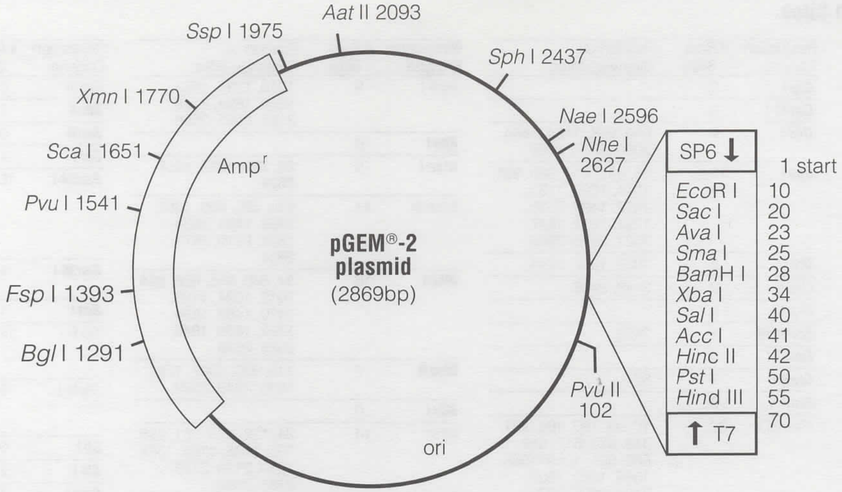


Figure 3. pGEM-2 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase initiation site 1
   b. T7 RNA polymerase initiation site 70
   c. SP6 RNA polymerase promoter 2852-2869
   d. T7 RNA polymerase promoter 71-87
   e. multiple cloning sites 10-60
   f. *lac* operon sequences 98-156
   g. β-lactamase (Amp^r) coding region 1098-1958
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pGEM-1 and -2 vectors are identical except for the orientation of the multiple cloning site region.

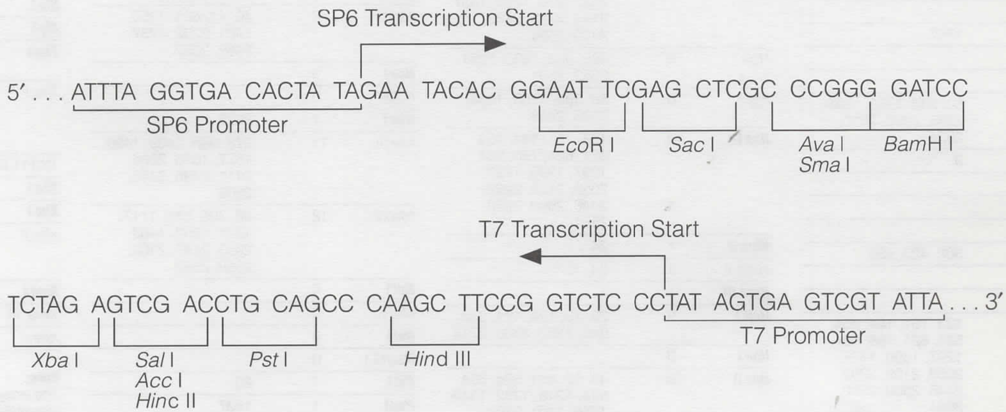


Figure 4. pGEM-2 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 4. pGEM-2 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2093
Acc I	1	41
Acc III	0	
Acy I	5	1708, 2090, 2448, 2562, 2583
Afl II	0	
Alu I	13	18, 57, 102, 220, 446, 536, 582, 839, 1360, 1460, 1523, 2202, 2221
Alw44 I	3	592, 1838, 2335
Apa I	0	
Asu I	6	1213, 1292, 1309, 1531, 2147, 2472
Ava I	1	23
Ava II	2	1309, 1531
Bal I	0	
BamH I	1	28
Ban I	4	1119, 2447, 2561, 2582
Ban II	3	20, 2514, 2528
Ban III	0	
Bbu I	1	2437
Bbv I	12	60, 89, 170, 188, 607, 697, 700, 906, 1209, 1600, 2211, 2393
Bcl I	0	
Bgl I	1	1291
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	20, 596, 1757, 1842, 2339, 2412, 2514, 2528
BspM I	1	53
BspM II	0	
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	18	127, 155, 188, 458, 525, 625, 799, 908, 1301, 1394, 1731, 2063, 2163, 2266, 2450, 2504, 2564, 2585
Cfr I	4	117, 1559, 2464, 2596
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	7	553, 962, 1128, 1668, 2094, 2329, 2693
Dpn I	16	30, 846, 921, 932, 940, 1018, 1030, 1135, 1476, 1494, 1540, 1798, 1815, 1851, 2531, 2622
Dra I	3	1037, 1056, 1748
Dra II	2	2147, 2472
Dra III	0	
Eco47 III	1	2503
Eco52 I	0	
EcoR I	1	10
EcoR V	0	
*Fnu*4H I	22	49, 103, 184, 202, 205, 323, 478, 621, 686, 689, 895, 1223, 1562, 1589, 1684, 1913, 2200, 2309, 2382, 2416, 2419, 2811
Fok I	4	1137, 1318, 1605, 2248
Fsp I	1	1393
Hae II	6	156, 526, 2451, 2505, 2565, 2586
Hae III	13	119, 293, 304, 322, 756, 1214, 1294, 1561, 2148, 2402, 2466, 2474, 2598
Hga I	6	389, 967, 1697, 2255, 2356, 2595
HgiA I	6	20, 596, 1757, 1842, 2339, 2412
Hha I	18	127, 155, 188, 458, 525, 625, 799, 908, 1301, 1394, 1731, 2063, 2163, 2266, 2450, 2504, 2564, 2585
Hinc II	1	42
Hind II	1	42
Hind III	1	55
Hinf I	9	38, 77, 113, 178, 253, 649, 1166, 2364, 2718
Hpa I	0	
Hpa II	18	24, 61, 485, 632, 658, 848, 1252, 1286, 1353, 1463, 1705, 2206, 2240, 2463, 2586, 2595, 2610, 2665
Hph I	9	1014, 1241, 1657, 1863, 1898, 2182, 2191, 2555, 2600
Kpn I	0	
Mae I	5	35, 773, 1026, 1361, 2628
Mae III	11	634, 697, 813, 1096, 1427, 1485, 1638, 1826, 2214, 2681, 2858
Mbo I	16	28, 844, 919, 930, 938, 1016, 1028, 1133, 1474, 1492, 1538, 1796, 1813, 1849, 2529, 2620
Mlu I	0	
Mnl I	14	42, 126, 177, 385, 460, 709, 1109, 1190, 1338, 1544, 2137, 2197, 2391, 2610
Msp I	18	24, 61, 485, 632, 658, 848, 1252, 1286, 1353, 1463, 1705, 2206, 2240, 2463, 2586, 2595, 2610, 2665
Mst I	1	1393
Nae I	1	2596
Nar I	3	2448, 2562, 2583
Nci I	9	24, 25, 658, 1354, 1705, 2206, 2241, 2463, 2666
Nco I	0	
Nde I	0	
Nhe I	1	2627
Nla III	11	282, 1002, 1493, 1503, 1581, 1617, 2010, 2115, 2199, 2437, 2510
Nla IV	12	30, 310, 349, 1121, 1215, 1256, 1467, 2057, 2449, 2473, 2563, 2584
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	50
Pvu I	1	1541
Pvu II	1	102
Rsa I	3	1651, 2327, 2791
Rsr II	0	
Sac I	1	20
Sac II	0	
Sal I	1	40
Sau3A I	16	28, 844, 919, 930, 938, 1016, 1028, 1133, 1474, 1492, 1538, 1796, 1813, 1849, 2529, 2620
Sau96 I	6	1213, 1292, 1309, 1531, 2147, 2472
Sca I	1	1651
ScrF I	12	24, 25, 306, 427, 440, 658, 1354, 1705, 2206, 2241, 2463, 2666
SfaN I	9	375, 1427, 1618, 1867, 2226, 2320, 2581, 2593, 2760
Sfi I	0	
Sin I	2	1309, 1531
Sma I	1	25
SnaB I	0	
Spe I	0	
Sph I	1	2437
Spo I	0	
Ssp I	1	1975
Sst I	1	20
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	14, 41, 91, 378, 1822, 2345
Tha I	11	125, 127, 325, 906, 1236, 1729, 2061, 2161, 2163, 2266, 2810
*Tth*111 II	3	868, 875, 907
Xba I	1	34
Xho I	0	
Xho II	8	28, 919, 930, 1016, 1028, 1796, 1813, 2620
Xma I	1	25
Xma III	0	
Xmn I	1	1770

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-3

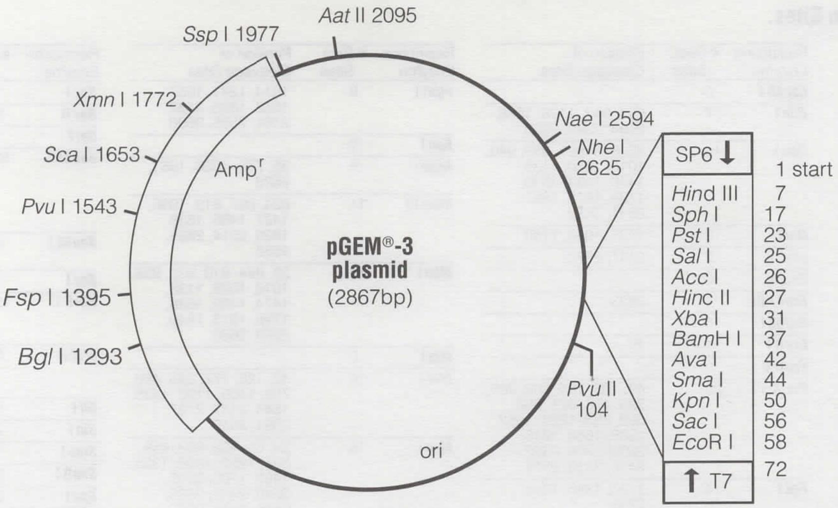


Figure 5. pGEM-3 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 72
   c. SP6 RNA polymerase promoter 2851-2867
   d. T7 RNA polymerase promoter 73-89
   e. multiple cloning sites 7-63
   f. *lac* operon sequences 100-158
   g. β-lactamase (Amp^r) coding region 1100-1960
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pGEM-3 and -4 vectors are identical except for the orientation of the multiple cloning site region.

SP6 Transcription Start

5′ . . . ATTTA GGTGA CACTA TAGAA TACAA GCTTG CATGC

SP6 Promoter — *Hind* III — *Sph* I

CTGCA GGTCG ACTCT AGAGG ATCCC CGGGT ACCGA GCTCG AATTC

Pst I — *Sal* I, *Acc* I, *Hinc* II — *Xba* I — *BamH* I — *Ava* I, *Sma* I — *Kpn* I — *Sac* I — *EcoR* I

T7 Transcription Start

CGGTC TCCCT ATAGT GAGTC GTATT A . . . 3′

T7 Promoter

Figure 6. pGEM-3 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 5. pGEM-3 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2095
Acc I	1	26
Acc III	0	
Acy I	5	1710, 2092, 2446, 2560, 2581
Afl II	0	
Aha II	5	1710, 2092, 2446, 2560, 2581
Alu I	13	9, 54, 104, 222, 448, 538, 584, 841, 1362, 1462, 1525, 2204, 2223
Alw44 I	3	594, 1840, 2337
Apa I	0	
Asu I	6	1215, 1294, 1311, 1533, 2149, 2470
Ava I	1	42
Ava II	2	1311, 1533
Bal I	0	
BamH I	1	37
Ban I	5	46, 1121, 2445, 2559, 2580
Ban II	3	56, 2512, 2526
Ban III	0	
Bbu I	1	17
Bbv I	11	91, 172, 190, 609, 699, 702, 908, 1211, 1602, 2213, 2395
Bcl I	0	
Bgl I	1	1293
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	56, 598, 1759, 1844, 2341, 2414, 2512, 2526
BspM I	1	12
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	3	308, 429, 442
BstX I	0	
Bsu36 I	0	
Cfo I	18	129, 157, 190, 460, 527, 627, 801, 910, 1303, 1396, 1733, 2065, 2165, 2268, 2448, 2502, 2562, 2583
Cfr I	4	119, 1561, 2462, 2594
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	7	555, 964, 1130, 1670, 2096, 2331, 2691
Dpn I	16	39, 848, 923, 934, 942, 1020, 1032, 1137, 1478. 1496, 1542, 1800,1817, 1853, 2529, 2620
Dra I	3	1039, 1058, 1750
Dra II	2	2149, 2470
Dra III	0	
Eco47 III	1	2501
Eco52 I	0	
EcoR I	1	58
EcoR V	0	
Fnu4H I	21	105, 186, 204, 207, 325, 480, 623, 688, 691, 897, 1225, 1564, 1591, 1686, 1915, 2202, 2311, 2384, 2418, 2421, 2809
Fok I	4	1139, 1320, 1607, 2250
Fsp I	1	1395
Hae II	6	158, 528, 2449, 2503, 2563, 2584
Hae III	13	121, 295, 306, 324, 758, 1216, 1296, 1563, 2150, 2404, 2464, 2472, 2596
Hga I	6	391, 969, 1699, 2257, 2358, 2593
HgiA I	6	56, 598, 1759, 1844, 2341, 2414
Hha I	18	129, 157, 190, 460, 527, 627, 801, 910, 1303, 1396, 1733, 2065, 2165, 2268, 2448, 2502, 2562, 2583
Hinc II	1	27
Hind II	1	27
Hind III	1	7
Hinf I	9	28, 79, 115, 180, 255, 651, 1168, 2366, 2716
Hpa I	0	
Hpa II	18	43, 63, 487, 634, 660, 850, 1254, 1288, 1355, 1465, 1707, 2208, 2242, 2461, 2584, 2593, 2608, 2663
Hph I	9	1016, 1243, 1659, 1865, 1900, 2184, 2193, 2553, 2598
Kpn I	1	50
Mae I	5	32, 775, 1028, 1363, 2626
Mae III	11	636, 699, 815, 1098, 1429, 1487, 1640, 1828, 2216, 2679, 2856
Mbo I	16	37, 846, 921, 932, 940, 1018, 1030, 1135, 1476, 1494, 1540, 1798, 1815, 1851, 2527, 2618
Mbo II	7	151, 942, 1013, 1768, 1846, 1955, 2522
Mlu I	0	
Mnl I	14	27, 128, 179, 387, 462, 711, 1111, 1192, 1340, 1546, 2139, 2199, 2393, 2608
Msp I	18	43, 63, 487, 634, 660, 850, 1254, 1288, 1355, 1465, 1707, 2208, 2242, 2461, 2584, 2593, 2608, 2663
Mst I	1	1395
Nae I	1	2594
Nar I	3	2446, 2560, 2581
Nci I	9	43, 44, 660, 1356, 1707, 2208, 2243, 2461, 2664
Nco I	0	
Nde I	0	
Nhe I	1	2625
Nla III	11	17, 284, 1004, 1495, 1505, 1583, 1619, 2012, 2117, 2201, 2508
Nla IV	13	39, 48, 312, 351, 1123, 1217, 1258, 1469, 2059, 2447, 2471, 2561, 2582
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	23
Pvu I	1	1543
Pvu II	1	104
Rsa I	4	48, 1653, 2329, 2789
Rsr II	0	
Sac I	1	56
Sac II	0	
Sal I	1	25
Sau3A I	16	37, 846, 921, 932, 940, 1018, 1030, 1135, 1476, 1494, 1540, 1798, 1815, 1851, 2527, 2618
Sau96 I	6	1215, 1294, 1311, 1533, 2149, 2470
Sca I	1	1653
ScrF I	12	43, 44, 308, 429, 442, 660, 1356, 1707, 2208, 2243, 2461, 2664
SfaN I	9	377, 1429, 1620, 1869, 2228, 2322, 2579, 2591, 2758
Sfi I	0	
Sin I	2	1311, 1533
Sma I	1	44
SnaB I	0	
Spe I	0	
Sph I	1	17
Spo I	0	
Ssp I	1	1977
Sst I	1	56
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	26, 56, 93, 380, 1824, 2347
Tha I	11	127, 129, 327, 908, 1238, 1731, 2063, 2163, 2165, 2268, 2808
Tth111 II	3	870, 877, 909
Xba I	1	31
Xho I	0	
Xho II	8	37, 921, 932, 1018, 1030, 1798, 1815, 2618
Xma I	1	42
Xma III	0	
Xmn I	1	1772

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-4

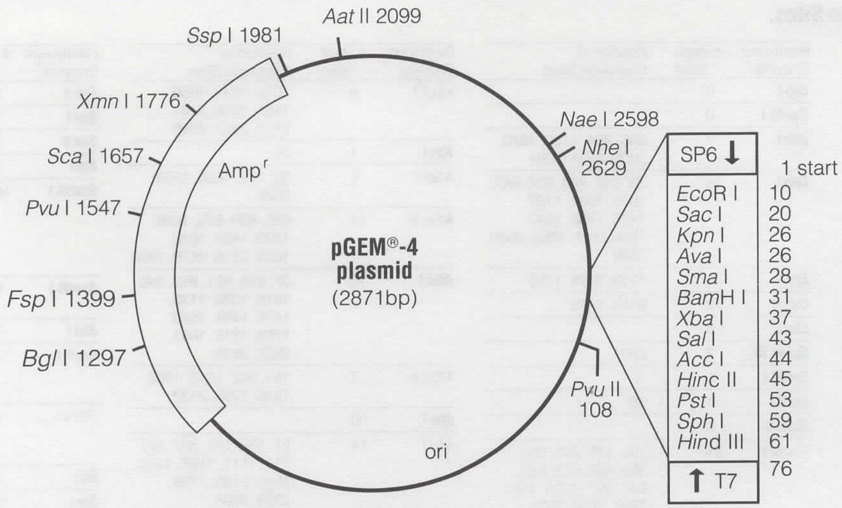


Figure 7. pGEM-4 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 76
   c. SP6 RNA polymerase promoter 2854-2871
   d. T7 RNA polymerase promoter 77-93
   e. multiple cloning sites 10-66
   f. *lac* operon sequences 104-162
   g. β-lactamase (Amp^r) coding region 1104-1964
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pGEM-3 and -4 vectors are identical except for the orientation of the multiple cloning site region.

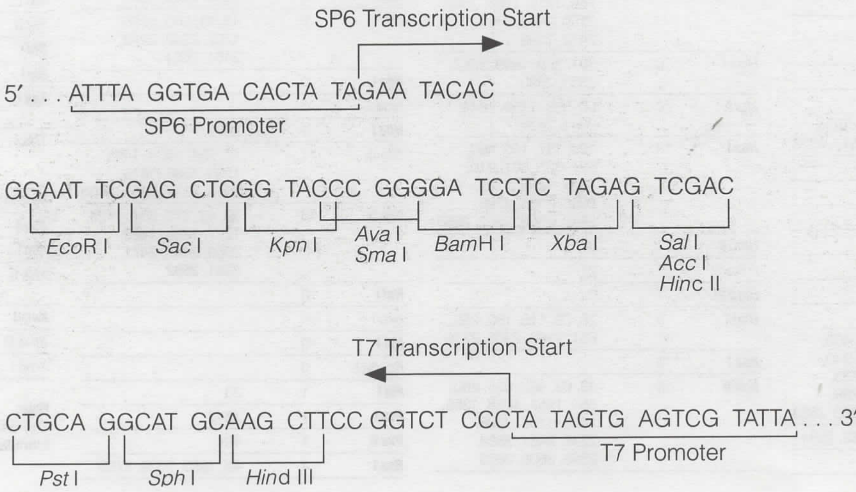


Figure 8. pGEM-4 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

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Table 6. pGEM-4 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
***Aat* II**	1	2099
***Acc* I**	1	44
***Acc* III**	0	
***Acy* I**	5	1714, 2096, 2450, 2564, 2585
Afl II	0	
Aha II	5	1714, 2096, 2450, 2564, 2585
***Alu* I**	12	18, 63, 108, 226, 452, 588, 845, 1366, 1466, 1529, 2208, 2227
***Alw44* I**	3	598, 1844, 2341
***Apa* I**	0	
***Asu* I**	6	1219, 1298, 1315, 1537, 2153, 2474
***Ava* I**	1	26
***Ava* II**	2	1315, 1537
Avr II	0	
***Bal* I**	0	
***BamH* I**	1	31
Ban I	5	22, 1125, 2449, 2563, 2584
***Ban* II**	3	20, 2516, 2530
***Bbu* I**	1	59
Bbv I	11	95, 176, 194, 613, 703, 706, 912, 1215, 1606, 2217, 2399
***Bcl* I**	0	
***Bgl* I**	1	1297
***Bgl* II**	0	
Bsm I	0	
***Bsp1286* I**	8	20, 602, 1763, 1848, 2345, 2418, 2516, 2530
BspM I	1	56
BspM II	0	
***BssH* II**	0	
***BstE* II**	0	
BstN I	3	312, 433, 446
***BstX* I**	0	
***Bsu36* I**	0	
***Cfo* I**	18	133, 161, 194, 464, 531, 631, 805, 914, 1307, 1400, 1737, 2069, 2169, 2272, 2452, 2506, 2566, 2587
***Cla* I**	0	
***Csp* I**	0	
***Csp45* I**	0	
***Dde* I**	7	559, 968, 1134, 1674, 2100, 2335, 2695
***Dpn* I**	16	33, 852, 927, 938, 946, 1024, 1036, 1141, 1482, 1500, 1546, 1804, 1821, 1857, 2533, 2624
***Dra* I**	3	1043, 1062, 1754
Dra II	2	2153, 2474
Dra III	0	
***Eco47* III**	1	2505
***Eco52* I**	0	
***EcoR* I**	1	10
***EcoR* V**	0	
Fnu4H I	21	109, 190, 208, 211, 329, 484, 627, 692, 695, 901, 1229, 1568, 1595, 1690, 1919, 2206, 2315, 2388, 2422, 2425, 2813
***Fok* I**	4	1143, 1324, 1611, 2254
Fsp I	1	1399
***Hae* II**	6	162, 532, 2453, 2507, 2567, 2588
***Hae* III**	13	125, 299, 310, 328, 762, 1220, 1300, 1567, 2154, 2408, 2468, 2476, 2600
Hga I	6	395, 973, 1703, 2261, 2362, 2597
HgiA I	6	20, 602, 1763, 1848, 2345, 2418
***Hha* I**	18	133, 161, 194, 464, 531, 631, 805, 914, 1307, 1400, 1737, 2069, 2169, 2272, 2452, 2506, 2566, 2587
***Hinc* II**	1	45
Hind II	1	45
***Hind* III**	1	61
***Hinf* I**	9	41, 83, 119, 184, 259, 655, 1172, 2370, 2720
***Hpa* I**	0	
***Hpa* II**	18	27, 67, 491, 638, 664, 854, 1258, 1292, 1359, 1469, 1711, 2212, 2246, 2465, 2588, 2597, 2612, 2667
Hph I	9	1020, 1247, 1663, 1869, 1904, 2188, 2197, 2557, 2602
***Kpn* I**	1	26
Mae I	5	38, 779, 1032, 1367, 2630
Mae II	4	987, 1403, 1776, 2096
Mae III	11	640, 703, 819, 1102, 1433, 1491, 1644, 1832, 2220, 2683, 2860
***Mbo* I**	16	31, 850, 925, 936, 944, 1022, 1034, 1139, 1480, 1498, 1544, 1802, 1819, 1855, 2531, 2622
Mbo II	7	155, 946, 1017, 1772, 1850, 1959, 2526
***Mlu* I**	0	
Mnl I	14	45, 132, 183, 391, 466, 715, 1115, 1196, 1344, 1550, 2143, 2203, 2397, 2612
***Msp* I**	18	27, 67, 491, 638, 664, 854, 1258, 1292, 1359, 1469, 1711, 2212, 2246, 2465, 2588, 2597, 2612, 2667
Mst II	0	
Nae I	1	2598
***Nar* I**	3	2450, 2564, 2585
Nci I	9	27, 28, 664, 1360, 1711, 2212, 2247, 2465, 2668
***Nhe* I**	1	2629
Nla III	11	59, 288, 1008, 1499, 1509, 1587, 1623, 2016, 2121, 2205, 2512
Nla IV	13	24, 33, 316, 355, 1127, 1221, 1262, 1473, 2063, 2451, 2475, 2565, 2586
***Not* I**	0	
Nru I	0	
***Nsi* I**	0	
PpuM I	0	
***Pst* I**	1	53
***Pvu* I**	1	1547
***Pvu* II**	1	108
***Rsa* I**	4	24, 1657, 2333, 2793
Rsr II	0	
***Sac* I**	1	20
***Sac* II**	0	
***Sal* I**	1	43
***Sau3A* I**	16	31, 850, 925, 936, 944, 1022, 1034, 1139, 1480, 1498, 1544, 1802, 1819, 1855, 2531, 2622
***Sau96* I**	6	1219, 1298, 1315, 1537, 2153, 2474
***Sca* I**	1	1657
ScrF I	12	27, 28, 312, 433, 446, 664, 1360, 1711, 2212, 2247, 2465, 2668
SfaN I	9	381, 1433, 1624, 1873, 2232, 2326, 2583, 2595, 2762
***Sfi* I**	0	
***Sin* I**	2	1315, 1537
***Sma* I**	1	28
***SnaB* I**	0	
***Spe* I**	0	
***Sph* I**	1	59
***Spo* I**	0	
***Ssp* I**	1	1981
Sst I	1	43
Sst II	0	
***Stu* I**	0	
***Sty* I**	0	
***Taq* I**	6	14, 44, 97, 384, 1828, 2351
Tha I	11	131, 133, 331, 912, 1242, 1735, 2067, 2167, 2169, 2272, 2812
Tth111 II	3	874, 881, 913
***Xba* I**	1	37
***Xho* I**	0	
Xho II	8	31, 925, 936, 1022, 1034, 1802, 1819, 2622
***Xma* I**	1	26
Xma III	0	
Xmn I	1	1776

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-3Z

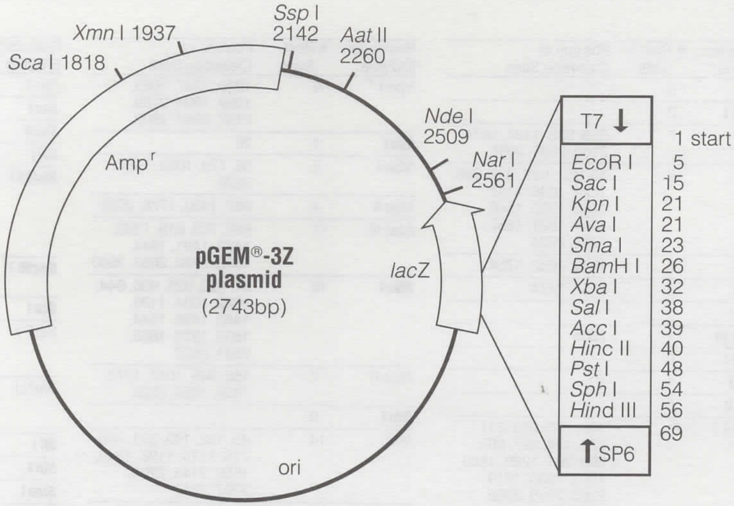


Figure 9. pGEM-3Z vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site — 1
   b. SP6 RNA polymerase transcription initiation site — 69
   c. T7 RNA polymerase promoter — 2727-2743
   d. SP6 RNA polymerase promoter — 70-86
   e. multiple cloning sites — 5-61
   f. *lacZ* start codon — 108
   g. *lac* operon sequences — 2561-2724; 94-323
   h. *lac* operator — 128-144
   i. β-lactamase (Amp^r) coding region — 1265-2125
   j. binding site of pUC/M13 forward sequencing primer — 2686-2702
   k. binding site of pUC/M13 reverse sequencing primer — 112-128
2. Specialized applications:
   a. blue/white screening for recombinants
   b. transcription *in vitro* from dual opposed promoters
3. The pGEM-3Z vector was previously called the pGEM-Blue vector.
4. The pGEM-3Z and -4Z vectors are identical except for the orientation of the SP6 and T7 promoters.

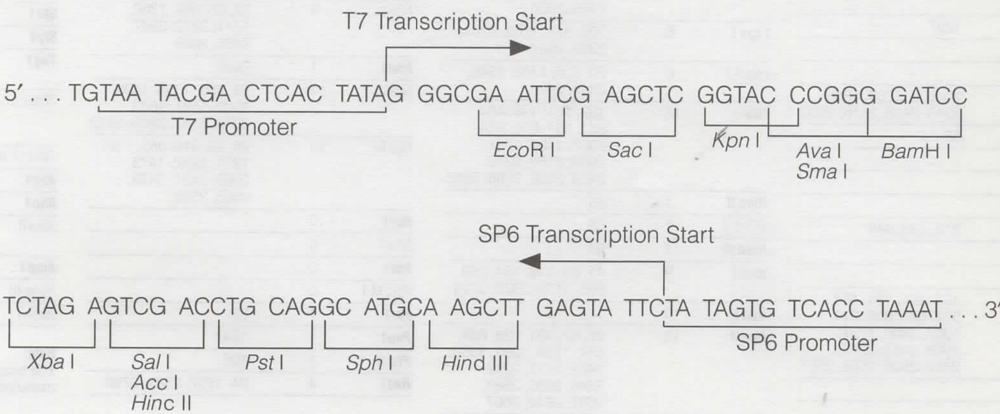


Figure 10. pGEM-3Z plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

Table 7. pGEM-3Z Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
***Aat* II**	1	2260
***Acc* I**	1	39
***Acc* III**	0	
***Acy* I**	3	1875, 2257, 2561
Afl II	0	
Aha I	0	
Aha II	3	1875, 2257, 2561
***Alu* I**	17	13, 58, 88, 110, 205, 269, 387, 613, 703, 749, 1006, 1527, 1627, 1690, 2369, 2388, 2633
***Alw44* I**	3	759, 2005, 2502
***Apa* I**	0	
***Asu* I**	6	1380, 1459, 1476, 1698, 2314, 2611
***Ava* I**	1	21
***Ava* II**	2	1476, 1698
***Bal* I**	0	
***BamH* I**	1	26
Ban I	4	17, 189, 1286, 2560
***Ban* II**	1	15
Ban III	0	
***Bbu* I**	1	54
Bbv I	12	256, 337, 355, 774, 864, 867, 1073, 1376, 1767, 2378, 2566, 2639
***Bcl* I**	0	
***Bgl* I**	2	1458, 2576
***Bgl* II**	0	
Bsm I	0	
***Bsp1286* I**	5	15, 763, 1924, 2009, 2506
BspM I	1	51
BspM II	0	
***BssH* II**	0	
***BstE* II**	0	
BstN I	5	185, 473, 594, 607, 2680
***BstX* I**	0	
***Bsu36* I**	0	
***Cfo* I**	17	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1898, 2230, 2330, 2433, 2563, 2584
Cfr I	3	284, 1726, 2713
***Cla* I**	0	
***Csp* I**	0	
***Csp45* I**	0	
***Dde* I**	6	720, 1129, 1295, 1835, 2261, 2496
***Dpn* I**	15	28, 1013, 1088, 1099, 1107, 1185, 1197, 1302, 1643, 1661, 1707, 1965, 1982, 2018, 2603
***Dra* I**	3	1204, 1223, 1915
Dra II	1	2314
Dra III	0	
***Eco47* III**	0	
***Eco52* I**	0	
***EcoR* I**	1	5
***EcoR* V**	0	
Fnu4H I	19	270, 351, 369, 372, 490, 645, 788, 853, 856, 1062, 1390, 1729, 1756, 1851, 2080, 2367, 2476, 2580, 2653
***Fok* I**	5	1304, 1485, 1772, 2415, 2659
Fsp I	2	1560, 2583
***Hae* I**	3	460, 471, 923
Hae II	3	323, 693, 2564
***Hae* III**	11	286, 460, 471, 489, 923, 1381, 1461, 1728, 2315, 2613, 2715
Hga I	4	556, 1134, 1864, 2422
HgiA I	5	15, 763, 1924, 2009, 2506
***Hha* I**	17	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1898, 2230, 2330, 2433, 2563, 2584
***Hinc* II**	1	40
Hind II	1	40
***Hind* III**	1	56
***Hinf* I**	7	36, 280, 345, 420, 816, 1333, 2733
***Hpa* I**	0	
***Hpa* II**	13	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1872, 2373, 2407
Hph I	8	69, 1181, 1408, 1824, 2030, 2065, 2349, 2358
***Kpn* I**	1	21
Mae I	4	33, 940, 1193, 1528
Mae II	5	1148, 1564, 1937, 2257, 2699
Mae III	12	75, 801, 864, 980, 1263, 1594, 1652, 1805, 1993, 2381, 2672, 2692
***Mbo* I**	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 2601
Mbo II	7	316, 1107, 1178, 1933, 2011, 2120, 2608
***Mlu* I**	0	
Mnl I	13	40, 293, 344, 552, 627, 876, 1276, 1357, 1505, 1711, 2304, 2364, 2624
***Msp* I**	13	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1872, 2373, 2407
Mst I	2	1560, 2583
Nae I	0	
***Nar* I**	1	2561
Nci I	7	22, 23, 825, 1521, 1872, 2373, 2408
***Nco* I**	0	
Nde I	1	2509
***Nhe* I**	0	
Nla III	11	54, 103, 449, 1169, 1660, 1670, 1748, 1784, 2177, 2282, 2366
Nla IV	11	19, 28, 191, 477, 516, 1288, 1382, 1423, 1634, 2224, 2562
***Not* I**	0	
Nru I	0	
***Nsi* I**	0	
PflM I	0	
PpuM I	0	
***Pst* I**	1	48
***Pvu* I**	2	1708, 2604
***Pvu* II**	2	269, 2633
***Rsa* I**	3	19, 1818, 2494
Rsr II	0	
***Sac* I**	1	15
***Sac* II**	0	
***Sal* I**	1	38
***Sau3A* I**	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 2601
***Sau96* I**	6	1380, 1459, 1476, 1698, 2314, 2611
***Sca* I**	1	1818
ScrF I	12	22, 23, 185, 473, 594, 607, 825, 1521, 1872, 2373, 2408, 2680
SfaN I	8	542, 1594, 1785, 2034, 2393, 2487, 2523, 2563
***Sfi* I**	0	
***Sin* I**	2	1476, 1698
***Sma* I**	1	23
***SnaB* I**	0	
***Spe* I**	0	
***Sph* I**	1	54
***Spo* I**	0	
***Ssp* I**	1	2142
Sst I	1	15
Sst II	0	
***Stu* I**	0	
***Sty* I**	0	
***Taq* I**	5	9, 39, 508, 545, 1989
Tha I	10	292, 294, 492, 1073, 1403, 1896, 2228, 2328, 2330, 2433
Tth111 II	3	1035, 1042, 1074
***Xba* I**	1	32
***Xho* I**	0	
Xho II	7	26, 1086, 1097, 1183, 1195, 1963, 1980
***Xma* I**	1	21
Xma III	0	
Xmn I	1	1937

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-4Z

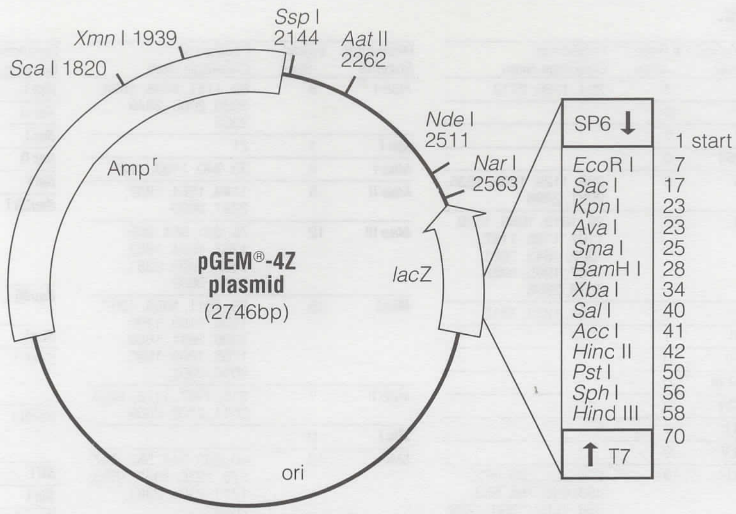


Figure 11. pGEM-4Z vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site — 1
   b. T7 RNA polymerase transcription initiation site — 70
   c. SP6 RNA polymerase promoter — 2729-2746
   d. T7 RNA polymerase promoter — 71-87
   e. multiple cloning sites — 7-63
   f. *lac*Z start codon — 110
   g. *lac* operon sequences — 2563-2726; 96-325
   h. *lac* operator — 130-146
   i. β-lactamase (Amp^r) coding region — 1267-2127
   j. binding site of pUC/M13 forward sequencing primer — 2684-2700
   k. binding site of pUC/M13 reverse sequencing primer — 110-126
2. Specialized applications:
   a. blue/white screening for recombinants
   b. transcription *in vitro* from dual opposed promoters
3. The pGEM-3Z and -4Z vectors are identical except for the orientation of the SP6 and T7 promoters.

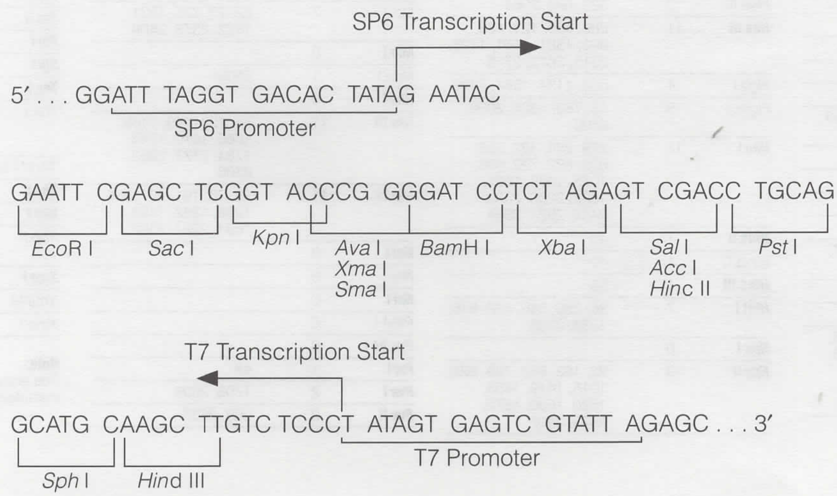


Figure 12. pGEM-4Z plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 8. pGEM-4Z Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2262
Acc I	1	41
Acc III	0	
Acy I	3	1877, 2259, 2563
Afl II	0	
Aha I	8	24, 25, 827, 1523, 1874, 2375, 2410
Aha II	3	1877, 2259, 2563
Alu I	17	15, 60, 90, 112, 207, 271, 389, 615, 705, 751, 1008, 1529, 1629, 1692, 2371, 2390, 2635
Alw44 I	3	761, 2007, 2504
Apa I	0	
Asu I	6	1382, 1461, 1478, 1700, 2316, 2613
Ava I	1	23
Ava II	2	1478, 1700
Bal I	0	
BamH I	1	28
Ban I	4	19, 191, 1288, 2562
Ban II	1	17
Ban III	0	
Bbu I	1	56
Bbv I	12	258, 339, 357, 776, 866, 869, 1075, 1378, 1769, 2380, 2568, 2641
Bcl I	0	
Bgl I	2	1460, 2578
Bgl II	0	
Bsm I	0	
Bsp1286 I	5	17, 765, 1926, 2011, 2508
BspM I	1	53
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	187, 475, 596, 609, 2682
BstX I	1	2725
Bsu36 I	0	
Cfo I	17	231, 296, 324, 357, 627, 694, 794, 968, 1077, 1470, 1563, 1900, 2232, 2332, 2435, 2565, 2586
Cfr I	3	286, 1728, 2715
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	722, 1131, 1297, 1837, 2263, 2498
Dpn I	15	30, 1015, 1090, 1101, 1109, 1187, 1199, 1304, 1645, 1663, 1709, 1967, 1984, 2020, 2605
Dra I	3	1206, 1225, 1917
Dra II	1	2316
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	1	7
EcoR V	0	
Fnu4H I	19	272, 353, 371, 374, 492, 647, 790, 855, 858, 1064, 1392, 1731, 1758, 1853, 2082, 2369, 2478, 2582, 2655
Fok I	5	1306, 1487, 1774, 2417, 2661
Fsp I	2	1562, 2585
Hae I	3	462, 473, 925
Hae II	3	325, 695, 2566
Hae III	11	288, 462, 473, 491, 925, 1383, 1463, 1730, 2317, 2615, 2717
Hga I	4	558, 1136, 1866, 2424
HgiA I	5	17, 765, 1926, 2011, 2508
Hha I	17	231, 296, 324, 357, 627, 694, 794, 968, 1077, 1470, 1563, 1900, 2232, 2332, 2435, 2565, 2586
Hinc II	1	42
Hind II	1	42
Hind III	1	58
Hinf I	8	38, 77, 248, 282, 347, 422, 818, 1335
Hpa I	0	
Hpa II	13	24, 165, 654, 801, 827, 1017, 1421, 1455, 1522, 1632, 1874, 2375, 2409
Hph I	7	1183, 1410, 1826, 2032, 2067, 2351, 2360
Kpn I	1	23
Mae I	4	35, 942, 1195, 1530
Mae II	5	1150, 1566, 1939, 2259, 2701
Mae III	12	803, 866, 982, 1265, 1596, 1654, 1807, 1995, 2383, 2674, 2694, 2735
Mbo I	15	28, 1013, 1088, 1099, 1107, 1185, 1197, 1302, 1643, 1661, 1707, 1965, 1982, 2018, 2603
Mbo II	7	318, 1109, 1180, 1935, 2013, 2122, 2610
Mlu I	0	
Mnl I	13	42, 295, 346, 554, 629, 878, 1278, 1359, 1507, 1713, 2306, 2366, 2626
Msp I	13	24, 165, 654, 801, 827, 1017, 1421, 1455, 1522, 1632, 1874, 2375, 2409
Mst I	2	1562, 2585
Nae I	0	
Nar I	1	2563
Nci I	7	24, 25, 827, 1523, 1874, 2375, 2410
Nco I	0	
Nde I	1	2511
Nhe I	0	
Nla III	11	56, 105, 451, 1171, 1662, 1672, 1750, 1786, 2179, 2284, 2368
Nla IV	11	21, 30, 193, 479, 518, 1290, 1384, 1425, 1636, 2226, 2564
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	1	50
Pvu I	2	1710, 2606
Pvu II	2	271, 2635
Rsa I	3	21, 1820, 2496
Rsr II	0	
Sac I	1	17
Sac II	0	
Sal I	1	40
Sau3A I	15	28, 1013, 1088, 1099, 1107, 1185, 1197, 1302, 1643, 1661, 1707, 1965, 1982, 2018, 2603
Sau96 I	6	1382, 1461, 1478, 1700, 2316, 2613
Sca I	1	1820
ScrF I	12	24, 25, 187, 475, 596, 609, 827, 1523, 1874, 2375, 2410, 2682
SfaN I	8	544, 1596, 1787, 2036, 2395, 2489, 2525, 2565
Sfi I	0	
Sin I	2	1478, 1700
Sma I	1	25
SnaB I	0	
Spe I	0	
Sph I	1	56
Spo I	0	
Ssp I	1	2144
Sst I	1	17
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	11, 41, 246, 510, 547, 1991
Tha I	10	294, 296, 494, 1075, 1405, 1898, 2230, 2330, 2332, 2435
Tth111 II	3	1037, 1044, 1076
Xba I	1	34
Xho I	0	
Xho II	7	28, 1088, 1099, 1185, 1197, 1965, 1982
Xma I	1	23
Xma III	0	
Xmn I	1	1939

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-3Zf(–)

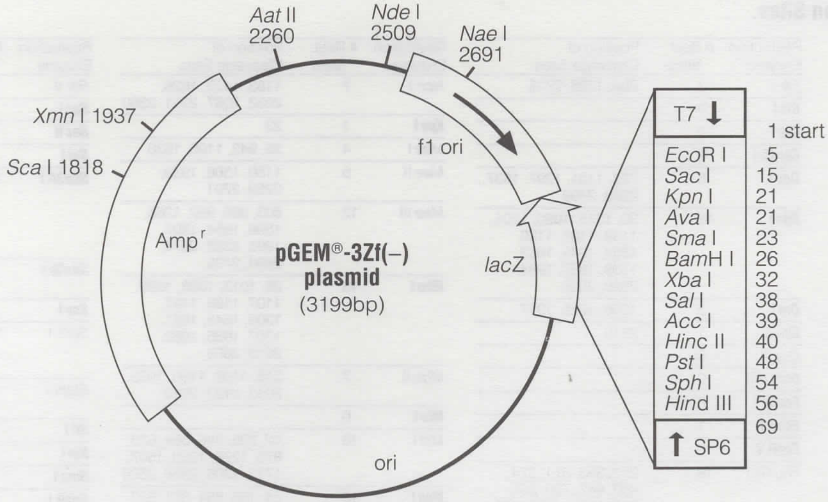


Figure 13. pGEM-3Zf(–) vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 69
   c. T7 RNA polymerase promoter 3183-3199
   d. SP6 RNA polymerase promoter 70-86
   e. multiple cloning sites 5-61
   f. *lac*Z start codon 108
   g. *lac* operon sequences 3020-3180; 94-323
   h. *lac* operator 128-144
   i. β-lactamase (Amp^r) coding region 1265-2125
   j. phage f1 region 2564-3019
   k. binding site of pUC/M13 forward sequencing primer 3140-3156
   l. binding site of pUC/M13 reverse sequencing primer 104-120
2. Specialized applications:
   a. ssDNA production
   b. blue/white screening for recombinants
   c. transcription *in vitro* from dual opposed promoters
3. The pGEM-3Zf(+) and -3Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the SP6 or pUC/M13 reverse primers to sequence ssDNA produced by the pGEM-3Zf(–) vector.

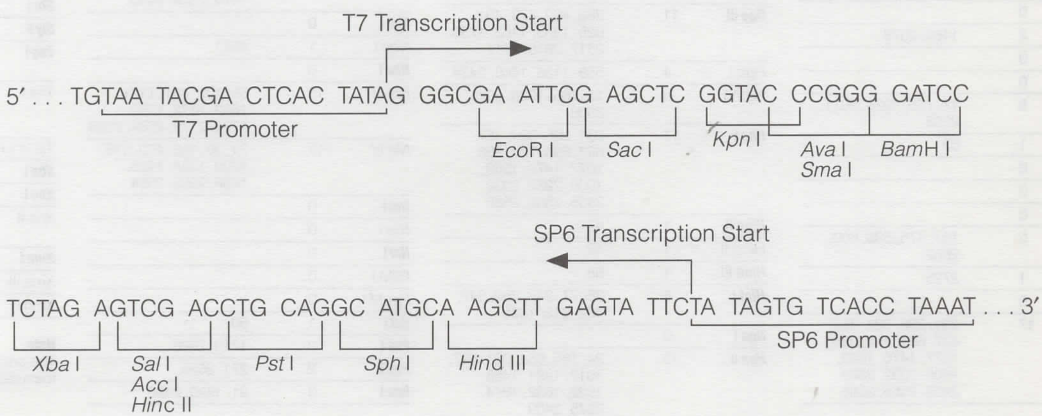


Figure 14. pGEM-3Zf(–) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is the same as the ssDNA strand produced by this vector.

Table 9. pGEM-3Zf(–) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2260
Acc I	1	39
Acc III	0	
Acy I	2	1875, 2257
Afl II	0	
Aha I	8	22, 23, 825, 1521, 1850, 1872, 2373, 2408
Aha II	2	1875, 2257
Alu I	20	13, 58, 88, 110, 205, 269, 387, 613, 703, 749, 1006, 1527, 1627, 1690, 2369, 2388, 2707, 2746, 2964, 3089
Alw44 I	3	759, 2005, 2502
Apa I	0	
Asu I	7	1380, 1459, 1476, 1698, 2314, 2800, 3067
Ava I	1	21
Ava II	2	1476, 1698
Bal I	0	
BamH I	1	26
Ban I	4	17, 189, 1286, 2753
Ban II	2	15, 2723
Ban III	0	
Bbu I	1	54
Bbv I	13	256, 337, 355, 774, 864, 867, 1073, 1376, 1767, 2378, 2624, 3022, 3095
Bcl I	0	
Bgl I	2	1458, 3032
Bgl II	0	
Bsm I	0	
Bsp1286 I	6	15, 763, 1924, 2009, 2506, 2723
BspM I	1	51
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	185, 473, 594, 607, 3136
BstX I	0	
Bsu36 I	0	
Cfo I	22	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1848, 2230, 2330, 2433, 2568, 2581, 2590, 2612, 2638, 2646, 3040
Cfr I	3	284, 1726, 3169
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	720, 1129, 1295, 1835, 2261, 2496
Dpn I	15	28, 1013, 1088, 1099, 1107, 1185, 1197, 1302, 1643, 1661, 1707, 1965, 1982, 2018, 3059
Dra I	3	1204, 1223, 1915
Dra II	1	2314
Dra III	1	2797
Eco47 III	0	
Eco52 I	0	
EcoR I	1	5
EcoR V	0	
Fnu4H I	21	270, 351, 369, 372, 490, 645, 788, 853, 856, 1062, 1390, 1729, 1756, 2080, 2367, 2476, 2577, 2591, 2613, 3036, 3109
Fok I	5	1304, 1485, 1772, 2415, 3115
Fsp I	2	1560, 3039
Hae I	3	460, 471, 923
Hae II	4	323, 693, 2639, 2647
Hae III	13	286, 460, 471, 489, 923, 1381, 1461, 1728, 2315, 2802, 2944, 3069, 3171
Hga I	5	556, 1134, 1864, 2422, 2572
HgiA I	5	15, 763, 1924, 2009, 2506
Hha I	22	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1848, 2230, 2330, 2433, 2568, 2581, 2590, 2612, 2638, 2646, 3040
Hinc II	1	40
Hind II	1	40
Hind III	1	56
Hinf I	9	36, 280, 345, 420, 816, 1333, 2842, 2864, 3189
Hpa I	0	
Hpa II	15	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1850, 1872, 2373, 2407, 2690
Hph I	9	69, 1181, 1408, 1824, 2030, 2065, 2349, 2358, 2794
Kpn I	1	21
Mae I	5	33, 940, 1193, 1528, 2641
Mae II	10	1148, 1564, 1937, 2257, 2683, 2793, 2836, 2848, 3007, 3155
Mae III	14	75, 801, 864, 980, 1263, 1594, 1652, 1805, 1993, 2381, 2604, 2616, 3128, 3148
Mbo I	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 3057
Mbo II	8	316, 1107, 1178, 1933, 2011, 2120, 2655, 3064
Mlu I	0	
Mnl I	14	40, 293, 344, 552, 627, 876, 1276, 1357, 1505, 1711, 2304, 2364, 2767, 3080
Msp I	14	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1872, 2373, 2407, 2690
Mst I	2	1560, 3039
Nae I	1	2691
Nar I	0	
Nci I	8	22, 23, 825, 1521, 1850, 1872, 2373, 2408
Nco I	0	
Nde I	1	2509
Nhe I	0	
Nla III	11	54, 103, 449, 1169, 1660, 1670, 1748, 1784, 2177, 2282, 2366
Nla IV	13	19, 28, 191, 477, 516, 1288, 1382, 1423, 1634, 2224, 2722, 2734, 2755
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	1	48
Pvu I	2	1708, 3060
Pvu II	2	269, 3089
Rsa I	3	19, 1818, 2494
Rsr II	0	
Sac I	1	15
Sac II	0	
Sal I	1	38
Sau3A I	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 3057
Sau96 I	7	1380, 1459, 1476, 1698, 2314, 2800, 3067
Sca I	1	1818
ScrF I	13	22, 23, 185, 473, 594, 607, 825, 1521, 1850, 1872, 2373, 2408, 3136
SfaN I	8	542, 1594, 1785, 2034, 2393, 2487, 2523, 2563
Sfi I	0	
Sin I	2	1476, 1698
Sma I	1	23
SnaB I	0	
Spe I	0	
Sph I	1	54
Spo I	0	
Ssp I	3	2142, 2978, 3002
Sst I	1	15
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	9, 39, 508, 545, 1989, 2759
Tha I	14	292, 294, 492, 1073, 1403, 1846, 2228, 2328, 2330, 2433, 2566, 2590, 2610, 2986
Tth111 II	3	1035, 1042, 1074
Xba I	1	32
Xho I	0	
Xho II	7	26, 1086, 1097, 1183, 1195, 1963, 1980
Xma I	1	21
Xma III	0	
Xmn I	1	1937

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-3Zf(+)

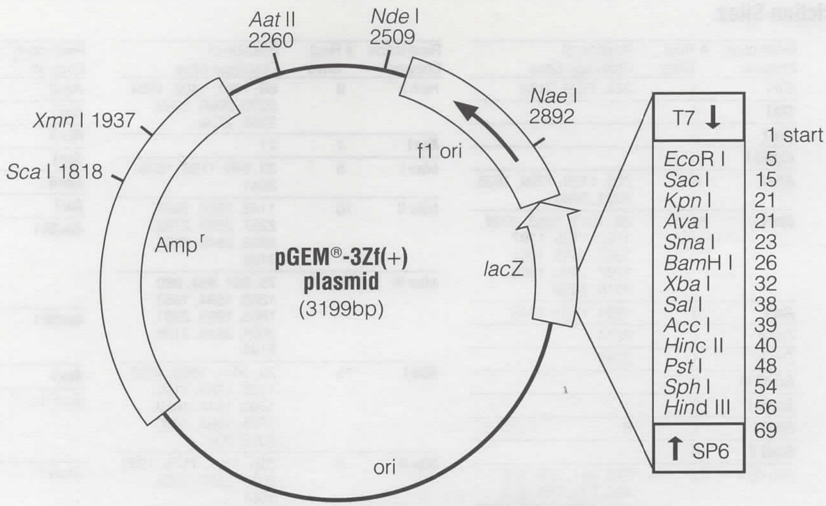


Figure 15. pGEM-3Zf(+) vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 69
   c. T7 RNA polymerase promoter 3183-3199
   d. SP6 RNA polymerase promoter 70-86
   e. multiple cloning sites 5-61
   f. *lac*Z start codon 108
   g. *lac* operon sequences 3020-3180; 94-323
   h. *lac* operator 128-144
   i. β-lactamase (Ampʳ) coding region 1265-2125
   j. phage f1 region 2564-3019
   k. binding site of pUC/M13 forward sequencing primer 3140-3156
   l. binding site of pUC/M13 reverse sequencing primer 104-120
2. Specialized applications:
   a. ssDNA production
   b. blue/white screening for recombinants
   c. transcription *in vitro* from dual opposed promoters
3. The pGEM-3Zf(+) and -3Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pGEM-3Zf(+) vector.

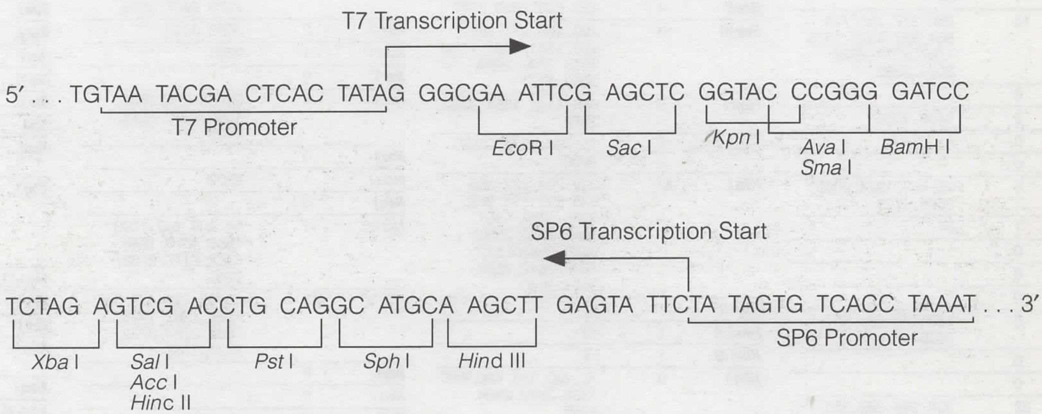


Figure 16. pGEM-3Zf(+) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA produced by this vector.

Table 10. pGEM-3Zf(+) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2260
Acc I	1	39
Acc III	0	
Acy I	2	1875, 2257
Afl II	0	
Aha I	8	22, 23, 825, 1521, 1850, 1872, 2373, 2408
Aha II	2	1875, 2257
Alu I	20	13, 58, 88, 110, 205, 269, 387, 613, 703, 749, 1006, 1527, 1627, 1690, 2369, 2388, 2618, 2836, 2875, 3089
Alw44 I	3	759, 2005, 2502
Apa I	0	
Asu I	7	1380, 1459, 1476, 1698, 2314, 2779, 3067
Ava I	1	21
Ava II	2	1476, 1698
Bal I	0	
BamH I	1	26
Ban I	4	17, 189, 1286, 2825
Ban II	2	15, 2863
Ban III	0	
Bbu I	1	54
Bbv I	13	256, 337, 355, 774, 864, 867, 1073, 1376, 1767, 2378, 2954, 3022, 3095
Bcl I	0	
Bgl I	2	1458, 3032
Bgl II	0	
Bsm I	0	
Bsp1286 I	6	15, 763, 1924, 2009, 2506, 2863
BspM I	1	51
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	185, 473, 594, 607, 3136
BstX I	0	
Bsu36 I	0	
Cfo I	22	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1848, 2230, 2330, 2433, 2938, 2946, 2972, 2994, 3003, 3016, 3040
Cfr I	3	284, 1726, 3169
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	720, 1129, 1295, 1835, 2261, 2496
Dpn I	15	28, 1013, 1088, 1099, 1107, 1185, 1197, 1302, 1643, 1661, 1707, 1965, 1982, 2018, 3059
Dra I	3	1204, 1223, 1915
Dra II	1	2314
Dra III	1	2788
Eco47 III	0	
Eco52 I	0	
EcoR I	1	5
EcoR V	0	
Fnu4H I	21	270, 351, 369, 372, 490, 645, 788, 853, 856, 1062, 1390, 1729, 1756, 2080, 2367, 2476, 2968, 2990, 3004, 3036, 3109
Fok I	5	1304, 1485, 1772, 2415, 3115
Fsp I	2	1560, 3039
Hae I	3	460, 471, 923
Hae II	4	323, 693, 2939, 2947
Hae III	13	286, 460, 471, 489, 923, 1381, 1461, 1728, 2315, 2638, 2780, 3069, 3171
Hga I	5	556, 1134, 1864, 2422, 3005
HgiA I	5	15, 763, 1924, 2009, 2506
Hha I	22	229, 294, 322, 355, 625, 692, 792, 966, 1075, 1468, 1561, 1848, 2230, 2330, 2433, 2938, 2946, 2972, 2994, 3003, 3016, 3040
Hinc II	1	40
Hind II	1	40
Hind III	1	56
Hinf I	9	36, 280, 345, 420, 816, 1333, 2715, 2737, 3189
Hpa I	0	
Hpa II	15	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1850, 1872, 2373, 2407, 2890
Hph I	9	69, 1181, 1408, 1824, 2030, 2065, 2349, 2358, 2789
Kpn I	1	21
Mae I	5	33, 940, 1193, 1528, 2939
Mae II	10	1148, 1564, 1937, 2257, 2573, 2732, 2744, 2787, 2897, 3155
Mae III	14	75, 801, 864, 980, 1263, 1594, 1652, 1805, 1993, 2381, 2961, 2973, 3128, 3148
Mbo I	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 3057
Mbo II	8	316, 1107, 1178, 1933, 2011, 2120, 2928, 3064
Mlu I	0	
Mnl I	14	40, 293, 344, 552, 627, 876, 1276, 1357, 1505, 1711, 2304, 2364, 2815, 3080
Msp I	14	22, 163, 652, 799, 825, 1015, 1419, 1453, 1520, 1630, 1872, 2373, 2407, 2890
Mst I	2	1560, 3039
Nae I	1	2891
Nar I	0	
Nci I	8	22, 23, 825, 1521, 1850, 1872, 2373, 2408
Nco I	0	
Nde I	1	2509
Nhe I	0	
Nla III	11	54, 103, 449, 1169, 1660, 1670, 1748, 1784, 2177, 2282, 2366
Nla IV	13	19, 28, 191, 477, 516, 1288, 1382, 1423, 1634, 2224, 2827, 2848, 2860
Not I	0	
Nru I	0	
Nsi I		
PflM I	0	
PpuM I	0	
Pst I	1	48
Pvu I	2	1708, 3060
Pvu II	2	269, 3089
Rsa I	3	19, 1818, 2494
Rsr II	0	
Sac I	1	15
Sac II	0	
Sal I	1	38
Sau3A I	15	26, 1011, 1086, 1097, 1105, 1183, 1195, 1300, 1641, 1659, 1705, 1963, 1980, 2016, 3057
Sau96 I	7	1380, 1459, 1476, 1698, 2314, 2779, 3067
Sca I	1	1818
ScrF I	13	22, 23, 185, 473, 594, 607, 825, 1521, 1850, 1872, 2373, 2408, 3136
SfaN I	8	542, 1594, 1785, 2034, 2393, 2487, 2523, 2563
Sfi I	0	
Sin I	2	1476, 1698
Sma I	1	23
SnaB I	0	
Spe I	0	
Sph I	1	54
Spo I	0	
Ssp I	3	2142, 2580, 2604
Sst I	1	15
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	6	9, 39, 508, 545, 1989, 2821
Tha I	14	292, 294, 492, 1073, 1403, 1846, 2228, 2328, 2330, 2433, 2596, 2972, 2992, 3016
Tth111 II	3	1035, 1042, 1074
Xba I	1	32
Xho I	0	
Xho II	7	26, 1086, 1097, 1183, 1195, 1963, 1980
Xma I	1	21
Xma III	0	
Xmn I	1	1937

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-5Zf(–)

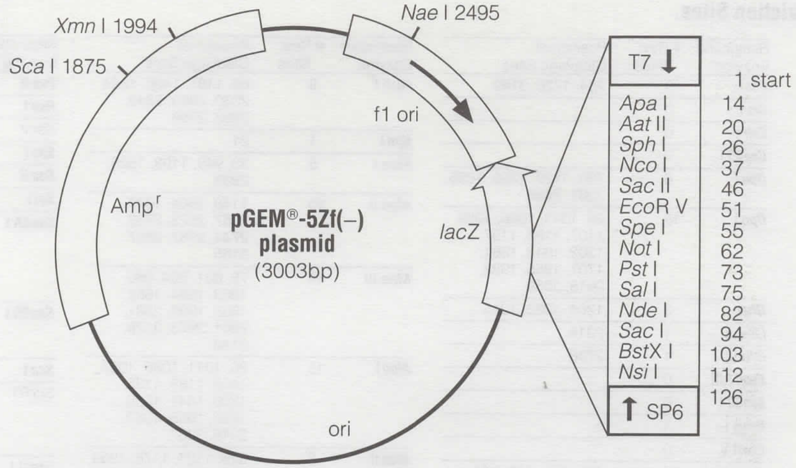


Figure 17. pGEM-5Zf(–) vector circle map.

Notes:

1. Sequence reference points:
   - a. T7 RNA polymerase transcription initiation site — 1
   - b. SP6 RNA polymerase transcription initiation site — 126
   - c. T7 RNA polymerase promoter — 2987-3003
   - d. SP6 RNA polymerase promoter — 127-143
   - e. multiple cloning sites — 10-113
   - f. *lacZ* start codon — 165
   - g. *lac* operon sequences — 2824-2984; 151-180
   - h. *lac* operator — 151-201
   - i. β-lactamase (Ampr) coding region — 1322-2182
   - j. phage f1 region — 2368-2823
   - k. binding site of pUC/M13 forward sequencing primer — 2944-2960
   - l. binding site of pUC/M13 reverse sequencing primer — 161-177
2. Specialized applications:
   - a. used with the Erase-a-Base® System
   - b. ssDNA production
   - c. blue/white screening for recombinants
   - d. transcription *in vitro* from dual opposed promoters
3. The pGEM-5Zf(+) and -5Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the SP6 or pUC/M13 reverse primers to sequence ssDNA produced by the pGEM-5Zf(–) vector.

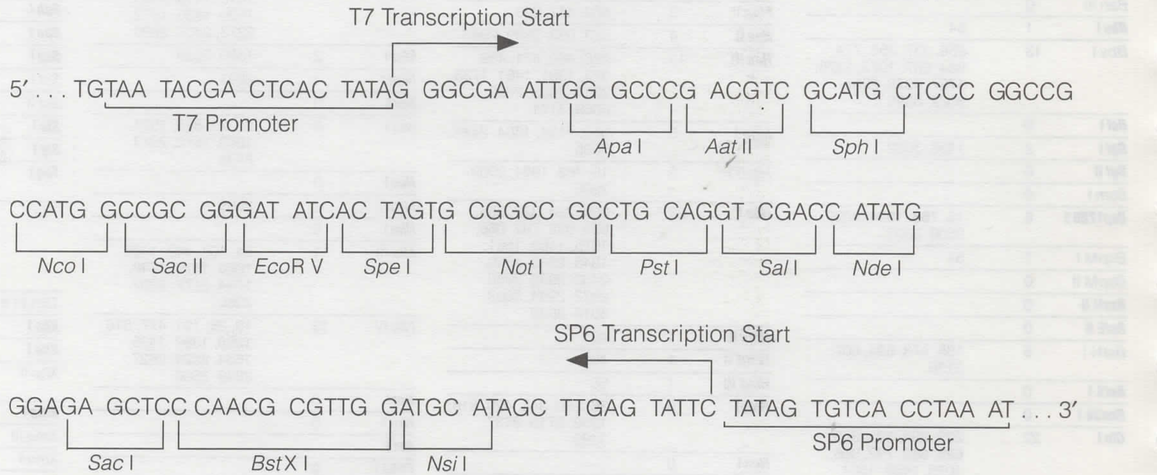


Figure 18. pGEM-5Zf(–) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is the same as the ssDNA strand produced by this vector.

Table 11. pGEM-5Zf(–) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	20
Acc I	1	76
Acc III	0	
Acy I	2	17, 1932
Aha II	2	17, 1932
Alu I	18	92, 115, 145, 167, 262, 326, 444, 670, 760, 806, 1063, 1584, 1684, 1747, 2511, 2550, 2768, 2893
Alw44 I	2	816, 2062
Apa I	1	14
Asu I	8	10, 11, 1437, 1516, 1533, 1755, 2604, 2871
Ava I	0	
Ava II	2	1533, 1755
Bal I	0	
BamH I	0	
Ban I	3	246, 1343, 2557
Ban II	3	14, 94, 2527
Ban III	0	
Bbu I	1	26
Bbv I	12	313, 394, 412, 831, 921, 924, 1130, 1433, 1824, 2428, 2826, 2899
Bcl I	0	
Bgl I	3	39, 1515, 2836
Bgl II	0	
Bsm I	0	
Bsp1286 I	6	14, 94, 820, 1981, 2066, 2527
BspM I	1	62
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	242, 530, 651, 664, 2940
BstX I	1	103
Bsu36 I	0	
Cfo I	20	286, 351, 379, 412, 682, 749, 849, 1023, 1132, 1525, 1618, 1905, 2287, 2372, 2385, 2394, 2416, 2442, 2450, 2844
Cfr I	6	31, 40, 62, 341, 1783, 2973
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	4	777, 1186, 1352, 1892
Dpn I	14	1070, 1145, 1156, 1164, 1242, 1254, 1359, 1700, 1718, 1764, 2022, 2039, 2075, 2863
Dra I	3	1261, 1280, 1972
Dra II	0	
Dra III	1	2601
Eco47 III	0	
Eco52 I	0	
EcoR I	0	
EcoR V	1	51
Fnu4H I	23	34, 43, 62, 65, 327, 408, 426, 429, 547, 702, 845, 910, 913, 1119, 1447, 1786, 1813, 2137, 2381, 2395, 2417, 2840, 2913
Fok I	5	119, 1361, 1542, 1829, 2919
Fsp I	2	1617, 2843
Hae I	3	517, 528, 980
Hae II	4	380, 750, 2443, 2451
Hae III	16	12, 33, 42, 64, 343, 517, 528, 546, 980, 1438, 1518, 1785, 2606, 2748, 2873, 2975
Hga I	4	613, 1191, 1921, 2376
HgiA I	4	94, 820, 1981, 2066
Hha I	20	286, 351, 379, 412, 682, 749, 849, 1023, 1132, 1525, 1618, 1905, 2287, 2372, 2385, 2394, 2416, 2442, 2450, 2844
Hinc II	1	77
Hind II	1	77
Hind III	0	
Hinf I	8	337, 402, 477, 873, 1390, 2646, 2668, 2993
Hpa I	0	
Hpa II	13	30, 220, 709, 856, 882, 1072, 1476, 1510, 1577, 1687, 1907, 1929, 2494
Hph I	7	126, 1238, 1465, 1881, 2087, 2122, 2598
Kpn I	0	
Mae I	5	56, 997, 1250, 1585, 2445
Mae II	10	17, 1205, 1621, 1994, 2487, 2597, 2640, 2652, 2811, 2959
Mae III	13	132, 858, 921, 1037, 1320, 1651, 1709, 1862, 2050, 2408, 2420, 2932, 2952
Mbo I	14	1068, 1143, 1154, 1162, 1240, 1252, 1357, 1698, 1716, 1762, 2020, 2037, 2073, 2861
Mbo II	8	373, 1164, 1235, 1990, 2068, 2177, 2459, 2868
Mlu I	1	99
Mnl I	11	350, 401, 609, 684, 933, 1333, 1414, 1562, 1768, 2571, 2884
Msp I	13	30, 220, 709, 856, 882, 1072, 1476, 1510, 1577, 1687, 1907, 1929, 2494
Nae I	1	2495
Nar I	0	
Nci I	5	30, 882, 1578, 1907, 1929
Nco I	1	37
Nde I	1	82
Nhe I	0	
Nla III	10	26, 41, 160, 506, 1226, 1717, 1727, 1805, 1841, 2234
Nla IV	12	12, 248, 534, 573, 1345, 1439, 1480, 1691, 2281, 2526, 2538, 2559
Not I	1	62
Nsi I	1	112
PflM I	0	
PpuM I	0	
Pst I	1	73
Pvu I	2	1765, 2864
Pvu II	2	326, 2893
Rsa I	1	1875
Rsr II	0	
Sac I	1	94
Sac II	1	46
Sal I	1	75
Sau3A I	14	1068, 1143, 1154, 1162, 1240, 1252, 1357, 1698, 1716, 1762, 2020, 2037, 2073, 2861
Sau96 I	8	10, 11, 1437, 1516, 1533, 1755, 2604, 2871
Sca I	1	1875
ScrF I	10	30, 242, 530, 651, 664, 882, 1578, 1907, 1929, 2940
SfaN I	7	97, 599, 1651, 1842, 2091, 2327, 2367
Sfi I	1	39
Sin I	2	1533, 1755
Sma I	0	
SnaB I	0	
Spe I	1	55
Sph I	1	26
Spo I	0	
Ssp I	3	2199, 2782, 2806
Sst I	1	94
Sst II	1	46
Stu I	0	
Sty I	1	37
Taq I	5	76, 565, 602, 2046, 2563
Tha I	13	45, 101, 349, 351, 549, 1130, 1460, 1903, 2285, 2370, 2394, 2414, 2790
Tth111 II	3	1092, 1099, 1131
Xba I	0	
Xho I	0	
Xho II	6	1143, 1154, 1240, 1252, 2020, 2037
Xma I	0	
Xma III	2	31, 62
Xmn I	1	1994

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-5Zf(+)

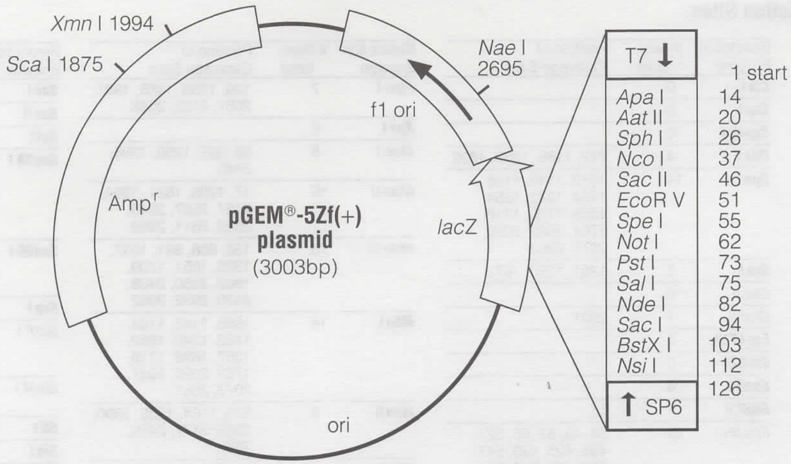


Figure 19. pGEM-5Zf(+) vector circle map.

Notes:

1. Sequence reference points:
   - a. T7 RNA polymerase transcription initiation site — 1
   - b. SP6 RNA polymerase transcription initiation site — 126
   - c. T7 RNA promoter — 2987-3003
   - d. SP6 RNA promoter — 127-143
   - e. multiple cloning sites — 10-113
   - f. *lacZ* start codon — 165
   - g. *lac* operon sequences — 2824-2984; 151-180
   - h. *lac* operator — 185-201
   - i. β-lactamase (Amp^r) coding region — 1322-2182
   - j. phage f1 region — 2368-2823
   - k. binding site of pUC/M13 forward sequencing primer — 2944-2960
   - l. binding site of pUC/M13 reverse sequencing primer — 161-177
2. Specialized applications:
   - a. used with the Erase-a-Base® System
   - b. ssDNA production
   - c. blue/white screening for recombinants
   - d. transcription *in vitro* from dual opposed promoters
3. The pGEM-5Zf(+) and -5Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pGEM-5Zf(+) vector.

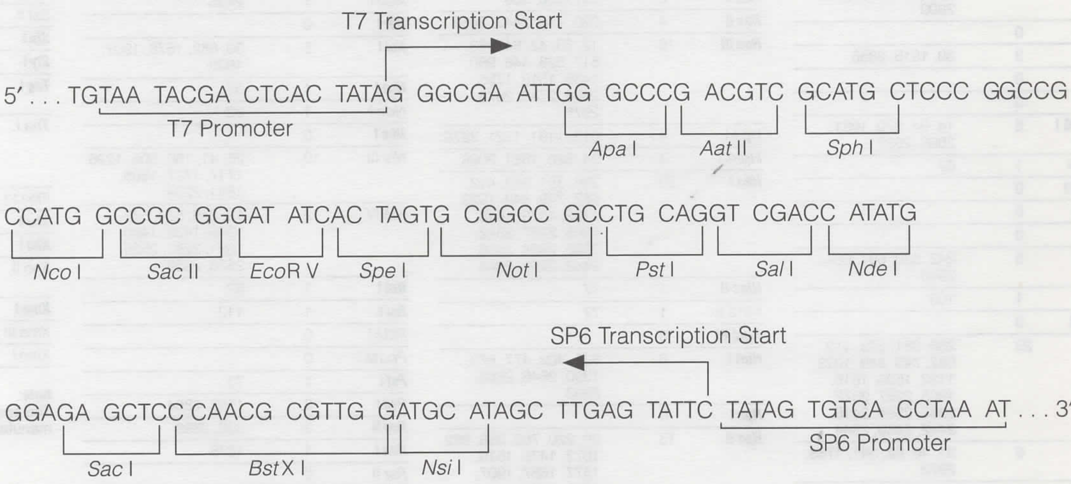


Figure 20. pGEM-5Zf(+) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA strand produced by this vector.

Promega

Table 12. pGEM-5Zf(+) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	20
Acc I	1	76
Acc III	0	
Acy I	2	17, 1932
Aha II	2	17, 1932
Alu I	18	92, 115, 145, 167, 262, 326, 444, 670, 760, 806, 1063, 1584, 1684, 1747, 2422, 2640, 2679, 2893
Alw44 I	2	816, 2062
Apa I	1	14
Asu I	8	10, 11, 1437, 1516, 1533, 1755, 2583, 2871
Ava I	0	
Ava II	2	1533, 1755
Bal I	0	
BamH I	0	
Ban I	3	246, 1343, 2629
Ban II	3	14, 94, 2667
Ban III	0	
Bbu I	1	26
Bbv I	12	313, 394, 412, 831, 921, 924, 1130, 1433, 1824, 2758, 2826, 2899
Bcl I	0	
Bgl I	3	39, 1515, 2836
Bgl II	0	
Bsm I	0	
Bsp1286 I	6	14, 94, 820, 1981, 2066, 2667
BspM I	1	62
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	242, 530, 651, 664, 2940
BstX I	1	103
Bsu 36 I	0	
Cfo I	20	286, 351, 379, 412, 682, 749, 849, 1023, 1132, 1525, 1618, 1905, 2287, 2742, 2750, 2776, 2798, 2807, 2820, 2844
Cfr I	6	31, 40, 62, 341, 1783, 2973
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	4	777, 1186, 1352, 1892
Dpn I	14	1070, 1145, 1156, 1164, 1242, 1254, 1359, 1700, 1718, 1764, 2022, 2039, 2075, 2863
Dra I	3	1261, 1280, 1972
Dra II	0	
Dra III	1	2592
Eco47 III	0	
Eco52 I	2	31, 26
EcoR I	0	
EcoR V	1	51
Fnu4H I	23	34, 43, 62, 65, 327, 408, 426, 429, 547, 702, 845, 910, 913, 1119, 1447, 1786, 1813, 2137, 2772, 2794, 2808, 2840, 2913
Fok I	5	119, 1361, 1542, 1829, 2919
Fsp I	2	1617, 2843
Hae I	3	517, 528, 980
Hae II	4	380, 750, 2743, 2751
Hae III	16	12, 33, 42, 64, 343, 517, 528, 546, 980, 1438, 1518, 1785, 2442, 2584, 2873, 2975
Hga I	4	613, 1191, 1921, 2809
HgiA I	4	94, 820, 1981, 2066
Hha I	20	286, 351, 379, 412, 682, 749, 849, 1023, 1132, 1525, 1618, 1905, 2287, 2742, 2750, 2776, 2798, 2807, 2820, 2844
Hinc II	1	77
Hind II	1	77
Hind III	0	
Hinf I	8	337, 402, 477, 873, 1390, 2519, 2541, 2993
Hpa I	0	
Hpa II	13	30, 220, 709, 856, 882, 1072, 1476, 1510, 1577, 1687, 1907, 1929, 2694
Hph I	7	126, 1238, 1465, 1881, 2087, 2122, 2593
Kpn I	0	
Mae I	5	56, 997, 1250, 1585, 2743
Mae II	10	17, 1205, 1621, 1994, 2377, 2536, 2548, 2591, 2701, 2959
Mae III	13	132, 858, 921, 1037, 1320, 1651, 1709, 1862, 2050, 2765, 2777, 2932, 2952
Mbo I	14	1068, 1143, 1154, 1162, 1240, 1252, 1357, 1698, 1716, 1762, 2020, 2037, 2073, 2861
Mbo II	8	373, 1164, 1235, 1990, 2068, 2177, 2732, 2868
Mlu I	1	99
Mnl I	11	350, 401, 609, 684, 933, 1333, 1414, 1562, 1768, 2619, 2884
Msp I	13	30, 220, 709, 856, 882, 1072, 1476, 1510, 1577, 1687, 1907, 1929, 2694
Nae I	1	2695
Nar I	0	
Nci I	5	30, 882, 1578, 1907, 1929
Nco I	1	37
Nde I	1	82
Nhe I	0	
Nla III	10	26, 41, 160, 506, 1226, 1717, 1727, 1805, 1841, 2234
Nla IV	12	12, 248, 534, 573, 1345, 1439, 1480, 1691, 2281, 2631, 2652, 2664
Not I	1	62
Nsi I	1	112
PflM I	0	
PpuM I	0	
Pst I	1	73
Pvu I	2	1765, 2864
Pvu II	2	326, 2893
Rsa I	1	1875
Rsr II	0	
Sac I	1	94
Sac II	1	46
Sal I	1	75
Sau3A I	14	1068, 1143, 1154, 1162, 1240, 1252, 1357, 1698, 1716, 1762, 2020, 2037, 2073, 2861
Sau96 I	8	10, 11, 1437, 1516, 1533, 1755, 2583, 2871
Sca I	1	1875
ScrF I	10	30, 242, 530, 651, 664, 882, 1578, 1907, 1929, 2940
SfaN I	7	97, 599, 1651, 1842, 2091, 2327, 2367
Sfi I	1	39
Sin I	2	1533, 1755
Sma I	0	
SnaB I	0	
Spe I	1	55
Sph I	1	26
Spo I	0	
Ssp I	3	2199, 2384, 2408
Sst I	1	94
Sst II	1	46
Stu I	0	
Sty I	1	37
Taq I	5	76, 565, 602, 2046, 2625
Tha I	13	45, 101, 349, 351, 549, 1130, 1460, 1903, 2285, 2400, 2776, 2796, 2820
Tth111 II	3	1092, 1099, 1131
Xba I	0	
Xho I	0	
Xho II	6	1143, 1154, 1240, 1252, 2020, 2037
Xma I	0	
Xma III	2	31, 62
Xmn I	1	1994

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-7Zf(–)

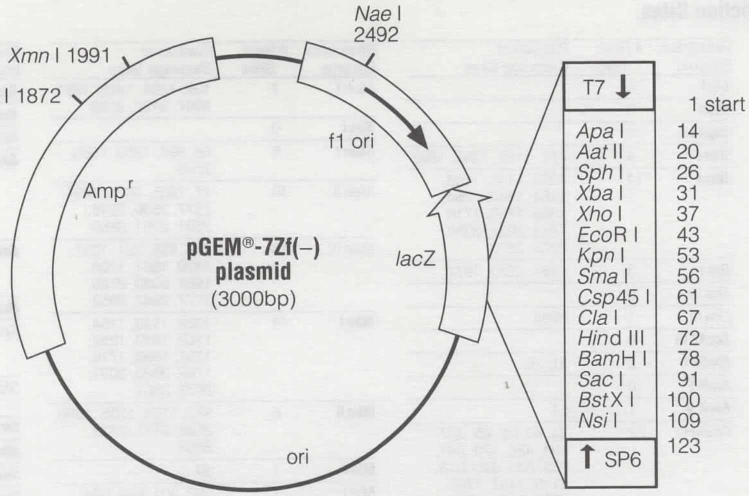


Figure 21. pGEM-7Zf(–) vector circle map.

Notes:

1. Sequence reference points:
   - a. T7 RNA polymerase transcription initiation site — 1
   - b. SP6 RNA polymerase transcription initiation site — 123
   - c. T7 RNA polymerase promoter — 2984-3000
   - d. SP6 RNA polymerase promoter — 124-140
   - e. multiple cloning sites — 10-110
   - f. *lacZ* start codon — 162
   - g. *lac* operon sequences — 2821-2981; 148-377
   - h. *lac* operator — 182-198
   - i. β-lactamase (Amp^r) coding region — 1319-2179
   - j. phage f1 region — 2365-2820
   - k. binding site of pUC/M13 forward sequencing primer — 2941-2957
   - l. binding site of pUC/M13 reverse sequencing primer — 158-174
2. Specialized applications:
   - a. used with the Erase-a-Base® System
   - b. ssDNA production
   - c. blue/white screening for recombinants
   - d. transcription *in vitro* from dual opposed promoters
3. The pGEM-7Zf(+) and -7Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the SP6 or pUC/M13 reverse primers to sequence ssDNA produced by the pGEM-7Zf(–) vector.

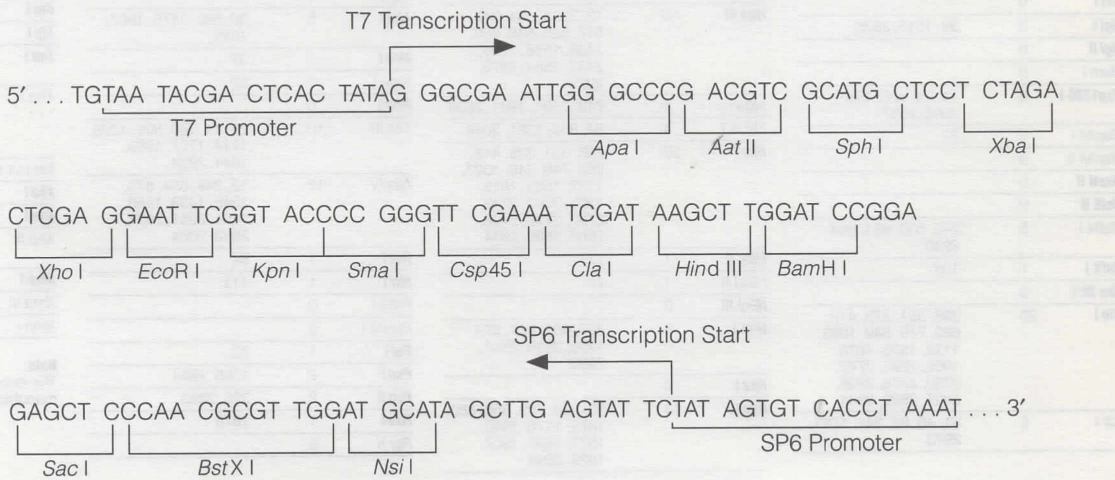


Figure 22. pGEM-7Zf(–) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is the same as the ssDNA strand produced by this vector.

Table 13. pGEM-7Zf(–) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
***Aat* II**	1	20
***Acc* I**	0	
***Acc* III**	1	81
***Acy* I**	2	17, 1929
Aha II	2	17, 1929
***Alu* I**	19	74, 89, 112, 142, 164, 259, 323, 441, 667, 757, 803, 1060, 1581, 1681, 1744, 2508, 2547, 2765, 2890
***Alw44* I**	2	813, 2059
***Apa* I**	1	14
***Asu* I**	8	10, 11, 1434, 1513, 1530, 1752, 2601, 2869
***Ava* I**	2	37, 54
***Ava* II**	2	1530, 1752
***Bal* I**	0	
***BamH* I**	1	78
Ban I	4	49, 243, 1340, 2554
***Ban* II**	3	14, 91, 2524
Ban III	1	67
***Bbu* I**	1	26
Bbv I	12	310, 391, 409, 828, 918, 921, 1127, 1430, 1821, 2425, 2823, 2896
***Bcl* I**	0	
***Bgl* I**	2	1512, 2833
***Bgl* II**	0	
Bsm I	0	
***Bsp1286* I**	6	14, 91, 817, 1978, 2063, 2524
BspM I	0	
BspM II	1	81
***BssH* II**	0	
***BstE* II**	0	
BstN I	5	239, 527, 648, 661, 2937
***BstX* I**	1	100
***Bsu36* I**	0	
***Cfo* I**	20	283, 348, 376, 409, 679, 746, 846, 1020, 1129, 1522, 1615, 1902, 2284, 2369, 2382, 2391, 2413, 2439, 2447, 2841
Cfr I	3	338, 1780, 2970
***Cla* I**	1	67
***Csp* I**	0	
***Csp45* I**	61	
***Dde* I**	4	774, 1183, 1349, 1889
***Dra* I**	3	1258, 1277, 1969
Dra II	0	
Dra III	1	2598
***Eco47* III**	0	
***Eco52* I**	0	
***EcoR* I**	1	43
***EcoR* V**	0	
Fnu4H I	19	324, 405, 423, 426, 544, 699, 842, 907, 910, 1116, 1444, 1783, 1810, 2134, 2378, 2392, 2414, 2837, 2910
***Fok* I**	5	116, 1358, 1539, 1826, 2916
Fsp I	2	1614, 2840
Hae I	3	514, 525, 977
***Hae* II**	4	377, 747, 2440, 2448
***Hae* III**	13	12, 340, 514, 525, 543, 977, 1435, 1515, 1782, 2603, 2745, 2870, 2972
Hga I	4	610, 1188, 1918, 2373
HgiA I	4	91, 817, 1978, 2063
***Hha* I**	20	283, 348, 376, 409, 679, 746, 846, 1020, 1129, 1522, 1615, 1902, 2284, 2369, 2382, 2391, 2413, 2439, 2447, 2841
***Hinc* II**	0	
Hind II	0	
***Hind* III**	1	72
***Hinf* I**	9	35, 334, 399, 474, 870, 1387, 2643, 2665, 2990
***Hpa* I**	0	
***Hpa* II**	14	55, 82, 217, 706, 853, 879, 1069, 1473, 1507, 1574, 1684, 1904, 1926, 2491
Hph I	7	123, 1235, 1462, 1878, 2084, 2119, 2595
***Kpn* I**	1	53
Mae I	5	32, 994, 1247, 1582, 2442
Mae II	10	17, 1202, 1618, 1991, 2484, 2594, 2637, 2649, 2808, 2956
Mae III	13	129, 855, 918, 1034, 1317, 1648, 1706, 1859, 2047, 2405, 2417, 2929, 2949
***Mbo* II**	8	370, 1161, 1232, 1987, 2065, 2174, 2456, 2865
***Mlu* I**	1	96
Mnl I	13	32, 39, 347, 398, 606, 681, 930, 1330, 1411, 1559, 1765, 2568, 2881
***Msp* I**	14	55, 82, 217, 706, 853, 879, 1069, 1473, 1507, 1574, 1684, 1904, 1926, 2491
Nae I	1	2492
***Nar* I**	0	
Nci I	6	55, 56, 879, 1575, 1904, 1926
***Nco* I**	0	
Nde I	0	
***Nhe* I**	0	
Nla III	9	26, 157, 503, 1223, 1714, 1724, 1802, 1838, 2231
Nla IV	14	12, 51, 80, 245, 531, 570, 1342, 1436, 1477, 1688, 2278, 2523, 2535, 2556
***Not* I**	0	
Nru I	0	
***Nsi* I**	1	109
PflM I	0	
PpuM I	0	
***Pst* I**	0	
***Pvu* I**	2	1762, 2861
***Pvu* II**	2	323, 2890
***Rsa* I**	2	51, 1872
Rsr II	0	
***Sac* I**	1	91
***Sac* II**	0	
***Sal* I**	0	
***Sau3A* I**	15	78, 1065, 1140, 1151, 1159, 1237, 1249, 1354, 1695, 1713, 1759, 2017, 2034, 2070, 2858
***Sau96* I**	8	10, 11, 1434, 1513, 1530, 1752, 2580, 2868
***Sca* I**	1	1872
ScrF I	11	55, 56, 239, 527, 648, 661, 879, 1575, 1904, 1926, 2937
SfaN I	7	94, 596, 1648, 1839, 2088, 2324, 2364
***Sfi* I**	0	
***Sin* I**	2	1530, 1752
***Sma* I**	1	56
***Spe* I**	0	
***Sph* I**	1	26
***Spo* I**	0	
***Ssp* I**	3	2196, 2779, 2803
Sst I	1	91
Sst II	0	
***Stu* I**	0	
***Sty* I**	0	
***Taq* I**	7	38, 61, 67, 562, 599, 2043, 2560
Tha I	12	98, 346, 348, 546, 1127, 1457, 1900, 2282, 2367, 2391, 2411, 2787
*Tth*111 II	3	1089, 1096, 1128
***Xba* I**	1	31
***Xho* I**	1	37
Xho II	7	78, 1140, 1151, 1237, 1249, 2017, 2034
***Xma* I**	1	54
Xma III	0	
Xmn I	1	1991

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-7Zf(+)

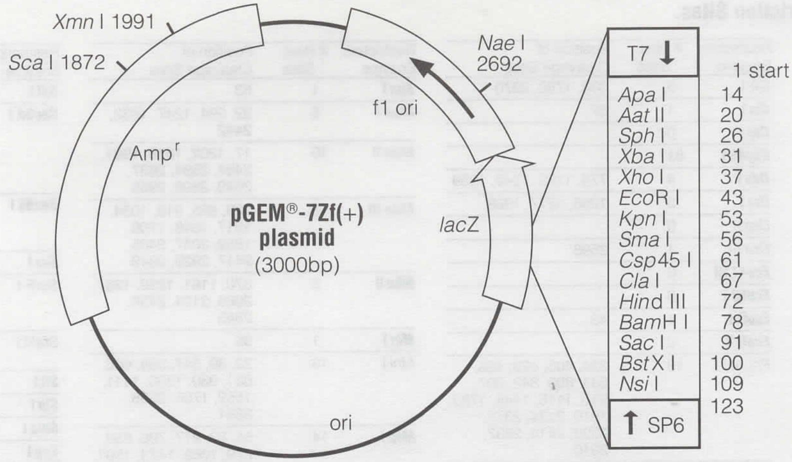


Figure 23. pGEM-7Zf(+) vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 123
   c. T7 RNA polymerase promoter 2984-3000
   d. SP6 RNA polymerase promoter 124-140
   e. multiple cloning sites 10-110
   f. *lacZ* start codon 162
   g. *lac* operon sequences 2821-2981; 148-377
   h. *lac* operator 182-198
   i. β-lactamase (Amp^r) coding region 1319-2179
   j. phage f1 region 2365-2820
   k. binding site of pUC/M13 forward sequencing primer 2941-2957
   l. binding site of pUC/M13 reverse sequencing primer 158-174
2. Specialized applications:
   a. used with the Erase-a-Base® System
   b. ssDNA production
   c. blue/white screening for recombinants
   d. transcription *in vitro* from dual opposed promoters
3. The pGEM-7Zf(+) and -7Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pGEM-7Zf(+) vector.

T7 Transcription Start

5′ . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCT CTAGA

T7 Promoter — *Apa* I — *Aat* II — *Sph* I — *Xba* I

CTCGA GGAAT TCGGT ACCCC GGGTT CGAAA TCGAT AAGCT TGGAT CCGGA

Xho I — *Eco*R I — *Kpn* I — *Sma* I — *Csp*45 I — *Cla* I — *Hind* III — *Bam*H I

SP6 Transcription Start

GAGCT CCCAA CGCGT TGGAT GCATA GCTTG AGTAT TCTAT AGTGT CACCT AAAT . . . 3′

Sac I — *Bst*X I — *Nsi* I — SP6 Promoter

Figure 24. pGEM-7Zf(+) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA strand produced by this vector.

Table 14. pGEM-7Zf(+) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	20
Acc I	0	
Acc III	1	81
Acy I	2	17, 1929
Aha II	2	17, 1929
Alu I	19	74, 89, 112, 142, 164, 259, 323, 441, 667, 757, 803, 1060, 1581, 1681, 1744, 2419, 2637, 2676, 2890
Alw 44 I	2	813, 2059
Apa I	1	14
Asu I	8	10, 11, 1434, 1513, 1530, 1752, 2580, 2869
Ava I	2	37, 54
Ava II	2	1530, 1752
Bal I	0	
BamH I	1	78
Ban I	4	49, 243, 1340, 2626
Ban II	3	14, 91, 2664
Ban III	1	67
Bbu I	1	26
Bbv I	12	310, 391, 409, 828, 918, 921, 1127, 1430, 1821, 2755, 2823, 2896
Bcl I	0	
Bgl I	2	1512, 2833
Bgl II	0	
Bsm I	0	
Bsp1286 I	6	14, 91, 817, 1978, 2063, 2664
BspM I	0	
BspM II	1	81
BssH II	0	
BstE II	0	
BstN I	5	239, 527, 648, 661, 2937
BstX I	1	100
Bsu36 I	0	
Cfo I	20	283, 348, 376, 409, 679, 746, 846, 1020, 1129, 1522, 1615, 1902, 2284, 2739, 2747, 2773, 2795, 2804, 2817, 2841
Cfr I	3	338, 1780, 2970
Cla I	1	67
Csp I	0	
Csp45 I	1	61
Dde I	4	774, 1183, 1349, 1889
Dra I	3	1258, 1277, 1969
Dra II	0	
Dra III	1	2589
Eco47 III	0	
Eco52 I	0	
EcoR I	1	43
EcoR V	0	
Fnu4H I	19	324, 405, 423, 426, 544, 699, 842, 907, 910, 1116, 1444, 1783, 1810, 2134, 2769, 2791, 2805, 2837, 2910
Fok I	5	116, 1358, 1539, 1826, 2916
Fsp I	2	1614, 2840
Hae I	3	514, 525, 977
Hae II	4	377, 747, 2740, 2748
Hae III	13	12, 340, 514, 525, 543, 977, 1435, 1515, 1782, 2439, 2581, 2870, 2972
Hga I	4	610, 1188, 1918, 2806
HgiA I	4	91, 817, 1978, 2063
Hha I	20	283, 348, 376, 409, 679, 746, 846, 1020, 1129, 1522, 1615, 1902, 2284, 2739, 2747, 2773, 2795, 2804, 2817, 2841
Hinc II	0	
Hind II	0	
Hind III	1	72
Hinf I	9	35, 334, 399, 474, 870, 1387, 2516, 2538, 2990
Hpa I	0	
Hpa II	14	55, 82, 217, 706, 853, 879, 1069, 1473, 1507, 1574, 1684, 1904, 1926, 2691
Hph I	7	123, 1235, 1462, 1878, 2084, 2119, 2590
Kpn I	1	53
Mae I	5	32, 994, 1247, 1582, 2740
Mae II	10	17, 1202, 1618, 1991, 2374, 2533, 2545, 2588, 2698, 2956
Mae III	13	129, 855, 918, 1034, 1317, 1648, 1706, 1859, 2047, 2762, 2774, 2929, 2949
Mbo II	8	370, 1161, 1232, 1987, 2065, 2174, 2729, 2865
Mlu I	1	96
Mnl I	13	32, 39, 347, 398, 606, 681, 930, 1330, 1411, 1559, 1765, 2616, 2881
Msp I	14	55, 82, 217, 706, 853, 879, 1069, 1473, 1507, 1574, 1684, 1904, 1926, 2691
Nae I	1	2692
Nar I	0	
Nci I	6	55, 56, 879, 1575, 1904, 1926
Nco I	0	
Nde I	0	
Nhe I	0	
Nla III	9	26, 157, 503, 1223, 1714, 1724, 1802, 1838, 2231
Nla IV	14	12, 51, 80, 245, 531, 570, 1342, 1436, 1477, 1688, 2278, 2628, 2649, 2661
Not I	0	
Nsi I	1	109
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	2	1762, 2861
Pvu II	2	323, 2890
Rsa I	2	51, 1872
Rsr II	0	
Sac I	1	91
Sac II	0	
Sal I	0	
Sau3A I	15	78, 1065, 1140, 1151, 1159, 1237, 1249, 1354, 1695, 1713, 1759, 2017, 2034, 2070, 2858
Sau96 I	8	10, 11, 1434, 1513, 1530, 1752, 2580, 2869
Sca I	1	1872
ScrF I	11	55, 56, 239, 527, 648, 661, 879, 1575, 1904, 1926, 2937
SfaN I	7	94, 596, 1648, 1839, 2088, 2324, 2364
Sfi I	0	
Sin I	2	1530, 1752
Sma I	1	56
Spe I	0	
Sph I	1	26
Spo I	0	
Ssp I	3	2196, 2381, 2405
Sst I	1	91
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	7	38, 61, 67, 562, 599, 2043, 2622
Tha I	12	98, 346, 348, 546, 1127, 1457, 1900, 2282, 2397, 2773, 2793, 2817
Tth111 II	3	1089, 1096, 1128
Xba I	1	31
Xho I	1	37
Xho II	7	78, 1140, 1151, 1237, 1249, 2017, 2034
Xma I	1	54
Xma III	0	
Xmn I	1	1991

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-9Zf(–)

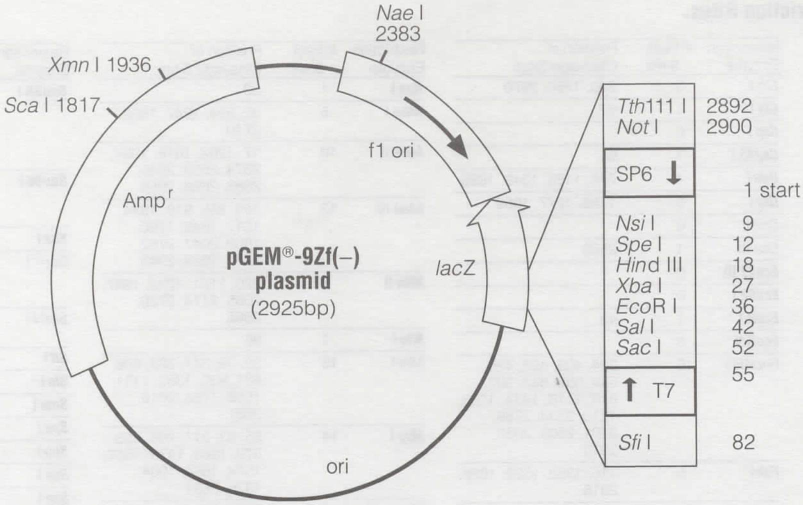


Figure 25. pGEM-9Zf(–) vector circle map.

Notes:

1. Sequence reference points:
   - a. SP6 RNA polymerase transcription initiation site — 1
   - b. T7 RNA polymerase transcription initiation site — 55
   - c. SP6 RNA polymerase promoter — 2909-2925
   - d. T7 RNA polymerase promoter — 56-72
   - e. multiple cloning sites — 5-54
   - f. *lacZ* start codon — 107
   - g. *lac* operon sequences — 93-22; 2724-2881
   - h. *lac* operator — 127-143
   - i. β-lactamase (Amp^r) coding region — 1264-2124
   - j. phage f1 region — 2256-2722
   - k. binding site of pUC/M13 forward sequencing primer — 2844-2860
   - l. binding site of pUC/M13 reverse sequencing primer — 103-119
2. Specialized applications:
   - a. allows excision of insert containing SP6 and T7 promoters
   - b. ssDNA production
   - c. blue/white screening for recombinants
   - d. transcription *in vitro* from dual opposed promoters
3. Use the T7 or pUC/M13 reverse primers to sequence ssDNA produced by the pGEM-9Zf(–) vector.

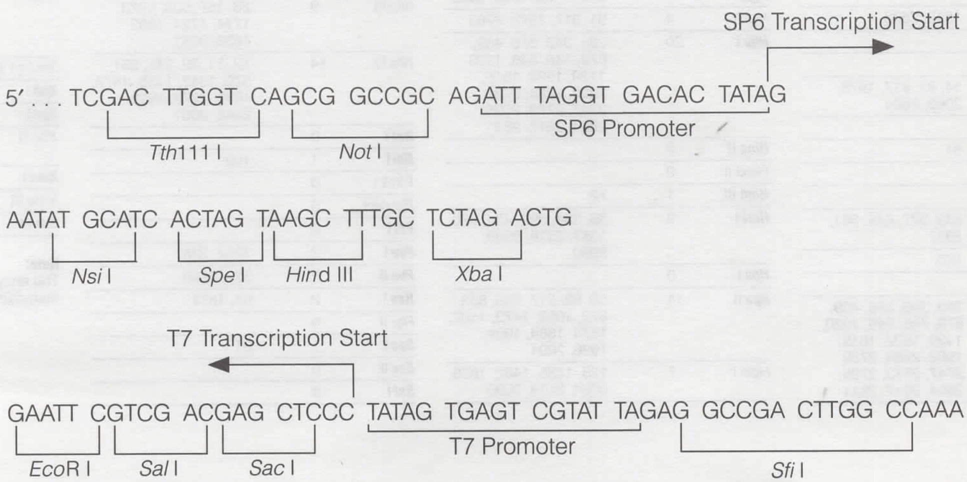


Figure 26. pGEM-9Zf(–) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase. The strand shown is the same as the ssDNA strand produced by this vector.

Table 15. pGEM-9Zf(–) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
***Aat* II**	0	
***Acc* I**	1	43
***Acc* III**	0	
***Acy* I**	1	1874
Afl II	0	
Aha I	0	
Aha II	1	1874
***Alu* I**	17	20, 50, 109, 204, 268, 386, 612, 702, 748, 1005, 1526 1626, 1689, 2399, 2656, 2793, 2886
***Alw44* I**	2	758, 2004
***Apa* I**	0	
***Asu* I**	6	1379, 1458, 1475, 1697, 2492, 2771
***Ava* I**	0	
***Ava* II**	2	1475, 1697
***Bal* I**	1	85
***BamH* I**	0	
Ban I	3	188, 1285, 2445
***Ban* II**	1	52
Ban III	0	
***Bbu* I**	0	
Bbv I	12	255, 336, 354, 773, 863, 866, 1072, 1375, 1766, 2316, 2726, 2799
***Bcl* I**	0	
***Bgl* I**	3	82, 1457, 2736
***Bgl* II**	3	82, 1457, 2736
***Bsp1286* I**	4	52, 762, 1923, 2008
BspM I	0	
BspM II	0	
***BssH* II**	0	
***BstE* II**	0	
BstN I	5	184, 472, 593, 606, 2840
***BstX* I**	0	
***Bsu36* I**	0	
***Cfo* I**	20	228, 293, 321, 354, 624, 691, 791, 965, 1074, 1467, 1560, 1897, 2229, 2260, 2273, 2282, 2304, 2330, 2338, 2744
Cfr I	5	83, 283, 1725, 2873, 2900
***Cla* I**	0	
***Csp* I**	0	
***Csp45* I**	0	
***Dde* I**	4	719, 1128, 1294, 1834
***Dra* I**	3	1203, 1222, 1914
Dra II	0	
Dra III	1	2489
***Eco47* III**	0	
***Eco52* I**	1	2900
***EcoR* I**	1	36
***EcoR* V**	0	
Fnu4H I	22	269, 350, 368, 371, 489, 644, 787, 852, 855, 1061, 1389, 1728, 1755, 1850, 2079, 2269, 2283, 2305, 2740, 2813, 2900, 2903
***Fok* I**	5	1303, 1484, 1771, 2399, 2819
Fsp I	2	1559, 2743
Hae I	4	85, 459, 470, 922
***Hae* II**	4	322, 692, 2331, 2339
***Hae* III**	15	76, 85, 285, 459, 470, 488, 922, 1380, 1460, 1727, 2494, 2636, 2773, 2875, 2902
Hga I	4	555, 1133, 1863, 2264
HgiA I	4	52, 762, 1923, 2008
***Hha* I**	20	228, 293, 321, 354, 624, 691, 791, 965, 1074, 1467, 1560, 1897, 2229, 2260, 2273, 2282, 2304, 2330, 2338, 2744
***Hinc* II**	1	44
***Hind* III**	1	18
***Hinf* I**	8	62, 279, 344, 419, 815, 1332, 2534, 2556
***Hpa* I**	0	
***Hpa* II**	11	162, 651, 798, 824, 1014, 1418, 1452, 1519, 1629, 1871, 2382
Hph I	6	1180, 1407, 1823, 2029, 2064, 2486
***Kpn* I**	0	
Mae I	6	13, 28, 939, 1192, 1527, 2333
Mae II	9	1147, 1563, 1936, 2375, 2485, 2528, 2540, 2710, 2859
Mae III	13	800, 863, 979, 1262, 1593, 1651, 1804, 1992, 2296, 2308, 2832, 2852, 2914
***Mbo* II**	8	315, 1106, 1177, 1932, 2010, 2119, 2347, 2768
***Mlu* I**	0	
Mnl I	12	65, 292, 343, 551, 626, 875, 1275, 1356, 1504, 1710, 2459, 2784
***Msp* I**	11	162, 651, 798, 824, 1014, 1418, 1452, 1519, 1629, 1871, 2382
Nae I	1	2383
***Nar* I**	0	
Nci I	3	824, 1520, 1871
***Nco* I**	0	
Nde I	0	
***Nhe* I**	0	
Nla III	8	102, 448, 1168, 1659, 1669, 1747, 1783, 2176
Nla IV	10	190, 476, 515, 1287, 1381, 1422, 1633, 2223, 2426, 2447
***Not* I**	1	2900
Nru I	0	
***Nsi* I**	1	9
PflM I	0	
PpuM I	0	
***Pst* I**	0	
***Pvu* I**	2	1707, 2764
***Pvu* II**	2	268, 2793
***Rsa* I**	1	1817
Rsr II	0	
***Sac* I**	1	52
***Sal* I**	1	42
***Sau3A* I**	14	1010, 1085, 1096, 1104, 1182, 1194, 1299, 1690, 1656, 1704, 1962, 1979, 2015, 2761
***Sau96* I**	6	1379, 1458, 1475, 1697, 2492, 2771
***Sca* I**	1	1817
ScrF I	8	184, 472, 493, 606, 824, 1520, 1871, 2840
SfaN I	6	16, 541, 1593, 1784, 2033, 2421
***Sfi* I**	1	82
***Sin* I**	2	1475, 1697
***Sma* I**	0	
***SnaB* I**	0	
***Spe* I**	1	12
***Sph* I**	0	
***Spo* I**	0	
***Ssp* I**	3	2141, 2694, 2705
Sst I	1	52
Sst II	0	
***Stu* I**	0	
***Sty* I**	0	
***Taq* I**	4	43, 544, 1988, 2451
Tha I	11	291, 293, 491, 1072, 1402, 1895, 2227, 2258, 2282, 2302, 2678
Tth111 I	1	2892
Tth111 II	3	1034, 1041, 1073
***Xba* I**	1	27
***Xho* I**	0	
Xho II	6	1085, 1096, 1182, 1194, 1962, 1979
***Xma* I**	0	
Xma III	1	2900
Xmn I	1	1936

Note:
The enzymes listed in boldface type are manufactured by Promega

Vector Maps

pGEM®-11Zf(–)

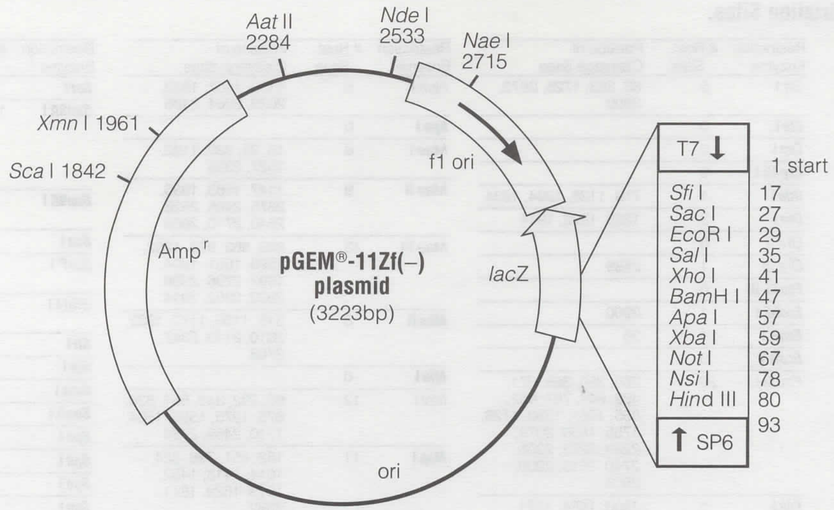


Figure 27. pGEM-11Zf(–) vector circle map.

Notes:

1. Sequence reference points:
   - a. T7 RNA polymerase transcription initiation site 1
   - b. SP6 RNA polymerase transcription initiation site 93
   - c. T7 RNA polymerase promoter 3207-3223
   - d. SP6 RNA polymerase promoter 94-110
   - e. multiple cloning sites 19-85
   - f. *lacZ* start codon 132
   - g. *lac* operon sequences 118-347; 3044-3204
   - h. *lac* operator 152-168
   - i. β-lactamase (Amp^r) coding region 1289-2149
   - j. phage f1 region 2588-3043
   - k. binding site of pUC/M13 forward sequencing primer 3164-3180
   - l. binding site of pUC/M13 reverse sequencing primer 128-144
2. Specialized applications:
   - a. *Sfi* I and *Not* I cut sites for subcloning from λgt11 *Sfi-Not* vector
   - b. ssDNA production
   - c. blue/white screening for recombinants
   - d. transcription *in vitro* from dual opposed promoters
3. The pGEM-11Zf(+) and -11Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the SP6 or pUC/M13 reverse primers to sequence ssDNA produced by the pGEM-11Zf(–) vector.

T7 Transcription Start

5′ . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG CCAAG TCGGCC

T7 Promoter — *Sfi* I

GAGC TCGAA TTCGT CGACC TCGAG GGATC CGGGCCC

Sac I — *EcoR* I — *Sal* I — *Xho* I — *BamH* I — *Apa* I

SP6 Transcription Start

TCT AGATG CGGCC GCATG CATAA GCTT GAGTA TTCTA TAGTG TCACC TAAAT . . . 3′

Xba I — *Not* I — *Nsi* I — *Hind* III — SP6 Promoter

Figure 28. pGEM-11Zf(–) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is the same as the ssDNA strand produced by this vector.

Table 16. pGEM-11Zf(–) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2284
Acc I	1	36
Acc III	0	
Acy I	2	1899, 2281
Afl II	0	
Aha I	0	
Aha II	2	1899, 2281
Alu I	20	25, 82, 112, 134, 229, 293, 411, 637, 727, 773, 1030, 1551, 1651, 1714, 2393, 2412, 2731, 2770, 2988, 3113
Alw44 I	3	783, 2029, 2526
Apa I	1	57
Asu I	9	53, 54, 1404, 1483, 1500, 1722, 2338, 2824, 3091
Ava I	1	41
Ava II	2	1500, 1722
Avr II	0	
Bal I	1	11
BamH I	1	47
Ban I	3	213, 1310, 2777
Ban II	3	27, 57, 2747
Ban III	0	
Bbu I	1	76
Bbv I	13	280, 361, 379, 798, 888, 891, 1097, 1400, 1791, 2402, 2648, 3046, 3119
Bcl I	0	
Bgl I	3	17, 1482, 3056
Bgl II	0	
Bsm I	0	
Bsp1286 I	7	27, 57, 787, 1948, 2033, 2530, 2747
BspM I	0	
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	209, 497, 618, 631, 3160
BstX I	0	
Bsu36 I	0	
Cfo I	22	253, 318, 346, 379, 649, 716, 816, 990, 1099, 1492, 1585, 1872, 2254, 2354, 2457, 2592, 2605, 2614, 2636, 2662, 2670, 3064
Cfr I	6	9, 18, 67, 308, 1750, 3193
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	744, 1153, 1319, 1859, 2285, 2520
Dpn I	15	49, 1037, 1112, 1123, 1131, 1209, 1221, 1326, 1667, 1685, 1731, 1989, 2006, 2042, 3083
Dra I	3	1228, 1247, 1939
Dra II	2	54, 2338
Dra III	1	2821
Eco47 III	0	
Eco52 I	2	18, 67
EcoR I	1	29
EcoR V	0	
Fnu4H I	23	67, 70, 294, 375, 393, 396, 514, 669, 812, 877, 880, 1086, 1414, 1753, 1780, 2104, 2391, 2500, 2601, 2615, 2637, 3060, 3133
Fok I	5	1328, 1509, 1796, 2439, 3139
Fsp I	2	1584, 3063
Hae II	4	347, 717, 2663, 2671
Hae III	17	11, 20, 55, 69, 310, 484, 495, 513, 947, 1405, 1485, 1752, 2339, 2826, 2968, 3093, 3195
Hga I	5	580, 1158, 1888, 2446, 2596
HgiA I	5	27, 787, 1948, 2033, 2530
Hha I	22	253, 318, 346, 379, 649, 716, 816, 990, 1099, 1492, 1585, 1872, 2254, 2354, 2457, 2592, 2605, 2614, 2636, 2662, 2670, 3064
Hinc II	1	37
Hind III	1	80
Hinf I	8	304, 369, 444, 840, 1357, 2866, 2888, 3213
Hpa I	0	
Hpa II	15	51, 187, 676, 823, 849, 1039, 1443, 1477, 1544, 1654, 1874, 1896, 2397, 2431, 2714
Hph I	9	93, 1205, 1432, 1848, 2054, 2089, 2373, 2382, 2818
Kpn I	0	
Mae I	5	60, 964, 1217, 1552, 2665
Mae II	10	1172, 1588, 1961, 2281, 2707, 2817, 2860, 2872, 3031, 3179
Mae III	14	99, 825, 888, 1004, 1287, 1618, 1676, 1829, 2017, 2405, 2628, 2640, 3152, 3172
Mbo II	8	340, 1131, 1202, 1957, 2035, 2144, 2679, 3088
Mlu I	0	
Mnl I	16	36, 50, 67, 317, 368, 576, 651, 900, 1300, 1381, 1529, 1735, 2328, 2388, 2791, 3104
Msp I	15	51, 187, 676, 823, 849, 1039, 1443, 1477, 1544, 1654, 1874, 1896, 2397, 2431, 2714
Nae I	1	2715
Nar I	0	
Nci I	7	52, 849, 1545, 1874, 1896, 2347, 2432
Nco I	0	
Nde I	1	2533
Nhe I	0	
Nla III	11	76, 127, 473, 1193, 1684, 1694, 1772, 1808, 2201, 2306, 2390
Nla IV	13	49, 55, 215, 501, 540, 1312, 1406, 1447, 1658, 2248, 2746, 2758, 2779
Not I	1	67
Nru I	0	
Nsi I	1	78
PpuM I	0	
Pst I	0	
Pvu I	2	1732, 3084
Pvu II	2	293, 3113
Rsa I	2	1842, 2518
Rsr II	0	
Sac I	1	27
Sac II	0	
Sal I	1	35
Sau3A I	15	47, 1035, 1110, 1121, 1129, 1207, 1219, 1324, 1665, 1683, 1729, 1987, 2004, 2040, 3081
Sau96 I	9	53, 54, 1404, 1483, 1500, 1722, 2338, 2824, 3091
Sca I	1	1842
ScrF I	12	52, 209, 497, 618, 631, 849, 1545, 1874, 1896, 2397, 2432, 3160
SfaN I	9	53, 566, 1618, 1809, 2058, 2417, 2511, 2547, 2587
Sfi I	1	17
Sin I	2	1500, 1722
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	1	76
Spo I	0	
Ssp I	3	2166, 3002, 3026
Sst I	1	27
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	7	27, 36, 42, 532, 569, 2013, 2783
Tha I	14	316, 318, 516, 1097, 1427, 1870, 2252, 2352, 2354, 2457, 2590, 2614, 2634, 3010
Tth111 I	0	
Tth111 II	3	1059, 1066, 1098
Xba I	1	59
Xho I	1	41
Xho II	7	47, 1110, 1121, 1207, 1219, 1987, 2004
Xma I	0	
Xma III	2	18, 67
Xmn I	1	1961

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-11Zf(+)

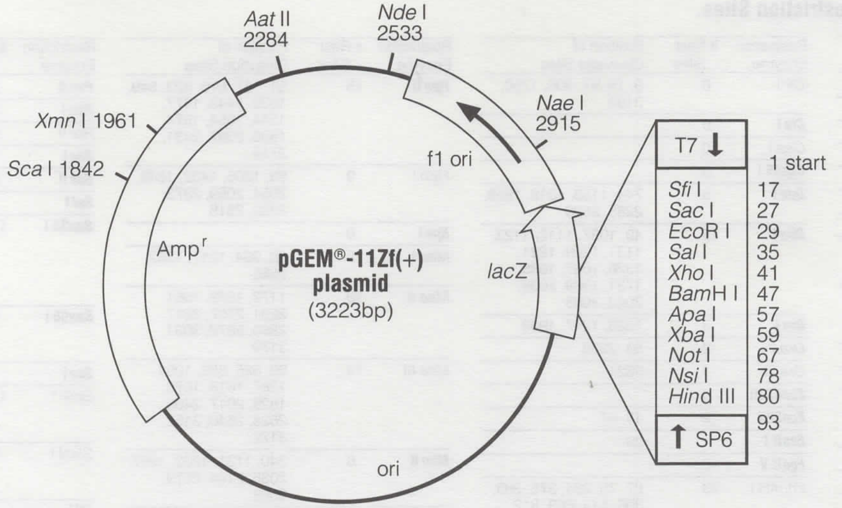


Figure 29. pGEM-11Zf(+) vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 93
   c. T7 RNA polymerase promoter 3207-3223
   d. SP6 RNA polymerase promoter 94-110
   e. multiple cloning sites 19-85
   f. *lac*Z start codon 132
   g. *lac* operon sequences 118-347; 3044-3204
   h. *lac* operator 152-168
   i. β-lactamase (Amp^r) coding region 1289-2149
   j. phage f1 region 2588-3043
   k. binding site of pUC/M13 forward sequencing primer 3164-3180
   l. binding site of pUC/M13 reverse sequencing primer 128-144
2. Specialized applications:
   a. *Sfi* I and *Not* I cut sites for subcloning from λgt11 *Sfi-Not* vector
   b. ssDNA production
   c. blue/white screening for recombinants
   d. transcription *in vitro* from dual opposed promoters
3. The pGEM-11Zf(+) and -11Zf(–) vectors are identical except for the orientation of the f1 origin for ssDNA production.
4. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pGEM-11Zf(+) vector.

T7 Transcription Start

5′ . . . TGTAA TACGA CTCAC TATAG GGCGA ATTGG CCAAG TCGGCC

T7 Promoter — *Sfi* I

GAGC TCGAA TTCGT CGACC TCGAG GGATC CGGGCCC

Sac I — *EcoR* I — *Sal* I — *Xho* I — *BamH* I — *Apa* I

SP6 Transcription Start

TCT AGATG CGGCC GCATG CATAA GCTTG AGTAT TCTAT AGTGT CACCT AAAT . . . 3′

Xba I — *Not* I — *Nsi* I — *Hind* III — SP6 Promoter

Figure 30. pGEM-11Zf(+) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA strand produced by this vector.

Table 17. pGEM-11Zf(+) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat I	1	2284
Acc I	1	36
Acc III	0	
Acy I	2	1899, 2281
Afl II	0	
Aha II	2	1899, 2281
Alu I	20	25, 82, 112, 134, 229, 293, 411, 637, 727, 773, 1030, 1551, 1651, 1714, 2393, 2412, 2642, 2860, 2899, 3113
Alw44 I	3	783, 2029, 2526
Apa I	1	57
Asu I	9	53, 54, 1404, 1483, 1500, 1722, 2338, 2824, 3091
Ava I	1	41
Ava II	2	1500, 1722
Avr II	0	
Bal I	1	11
BamH I	1	47
Ban I	3	213, 1310, 2849
Ban II	3	27, 57, 2887
Ban III	0	
Bbu I	1	76
Bbv I	13	280, 361, 379, 798, 888, 891, 1097, 1400, 1791, 2402, 2978, 3046, 3119
Bcl I	0	
Bgl I	3	17, 1482, 3056
Bgl II	0	
Bsm I	0	
Bsp1286 I	7	27, 57, 787, 1948, 2033, 2530, 2887
BspM I	0	
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	5	209, 497, 618, 631, 3160
BstX I	0	
Bsu36 I	0	
Cfo I	22	253, 318, 346, 379, 649, 716, 816, 990, 1099, 1492, 1585, 1872, 2254, 2354, 2457, 2962, 2970, 2996, 3018, 3027, 3040, 3064
Cfr I	6	9, 18, 67, 308, 1750, 3193
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	744, 1153, 1319, 1859, 2285, 2520
Dpn I	15	49, 1037, 1112, 1123, 1131, 1209, 1221, 1326, 1667, 1685, 1731, 1989, 2006, 2042, 3083
Dra I	3	1228, 1247, 1939
Dra II	2	54, 2338
Dra III	1	2812
Eco47 III	0	
Eco52 I	2	18, 67
EcoR I	1	29
EcoR V	0	
*Fnu*4H I	23	67, 70, 294, 375, 393, 396, 514, 669, 812, 877, 880, 1086, 1414, 1753, 1780, 2104, 2391, 2500, 2992, 3014, 3028, 3060, 3133
Fok I	5	1328, 1509, 1796, 2439, 3139
Fsp I	2	1584, 3063
Hae II	4	347, 717, 2963, 2971
Hae III	17	11, 20, 55, 69, 310, 484, 495, 513, 947, 1405, 1485, 1752, 2339, 2662, 2804, 3093, 3195
Hga I	5	580, 1158, 1888, 2446, 3029
HgiA I	5	27, 787, 1948, 2033, 2530
Hha I	22	253, 318, 346, 379, 649, 716, 816, 990, 1099, 1492, 1585, 1872, 2554, 2354, 2457, 2962, 2970, 2996, 3018, 3027, 3040, 3064
Hinc II	1	37
Hind III	1	80
Hinf I	8	304, 369, 444, 840, 1357, 2739, 2761, 3213
Hpa I	0	
Hpa II	15	51, 187, 676, 823, 849, 1039, 1443, 1477, 1544, 1654, 1874, 1896, 2397, 2431, 2914
Hph I	9	93, 1205, 1432, 1848, 2054, 2089, 2373, 2382, 2813
Kpn I	0	
Mae I	5	60, 964, 1217, 1552, 2963
Mae II	10	1172, 1588, 1961, 2281, 2597, 2756, 2768, 2811, 2921, 3179
Mae III	14	99, 825, 888, 1004, 1287, 1618, 1676, 1829, 2017, 2405, 2628, 2640, 3152, 3172
Mbo II	8	340, 1131, 1202, 1957, 2035, 2144, 2952, 3088
Mlu I	0	
Mnl I	16	36, 50, 67, 317, 368, 576, 651, 900, 1300, 1381, 1529, 1735, 2328, 2388, 2839, 3104
Msp I	15	51, 187, 676, 823, 849, 1039, 1443, 1477, 1544, 1654, 1874, 1896, 2397, 2431, 2914
Mst I	2	1584, 3063
Nae I	1	2915
Nar I	0	
Nci I	7	52, 849, 1545, 1874, 1896, 2397, 2432
Nco I	0	
Nde I	1	2533
Nhe I	0	
Nla III	11	76, 127, 473, 1193, 1684, 1694, 1772, 1808, 2201, 2306, 2390
Nla IV	13	49, 55, 215, 501, 540, 1312, 1406, 1447, 1658, 2248, 2851, 2872, 2884
Not I	1	67
Nru I	0	
Nsi I	1	78
PpuM I	0	
Pst I	0	
Pvu I	2	1732, 3084
Pvu II	2	293, 3113
Rsa I	2	1842, 2518
Rsr II	0	
Sac I	1	27
Sac II	0	
Sal I	1	35
Sau3A I	15	47, 1035, 1110, 1121, 1129, 1207, 1219, 1324, 1665, 1683, 1729, 1987, 2004, 2040, 3081
Sau96 I	9	53, 54, 1404, 1483, 1500, 1722, 2338, 2803, 3091
Sca I	1	1842
ScrF I	12	52, 209, 497, 618, 631, 849, 1545, 1874, 1896, 2397, 2432, 3160
SfaN I	9	53, 566, 1618, 1809, 2058, 2417, 2511, 2547, 2587
Sfi I	1	17
Sin I	2	1500, 1722
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	1	76
Spo I	0	
Ssp I	3	2166, 2604, 2628
Sst I	1	27
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	7	27, 36, 42, 532, 569, 2013, 2783
Tha I	14	316, 318, 516, 1097, 1427, 1870, 2252, 2352, 2354, 2457, 2620, 2996, 3016, 3040
*Tth*111 I	0	
*Tth*111 II	3	1059, 1066, 1098
Xba I	1	59
Xho I	1	41
Xho II	7	47, 1110, 1121, 1207, 1219, 1987, 2004
Xma I	0	
Xma III	2	18, 67
Xmn I	1	1961

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEM®-13Zf(+)

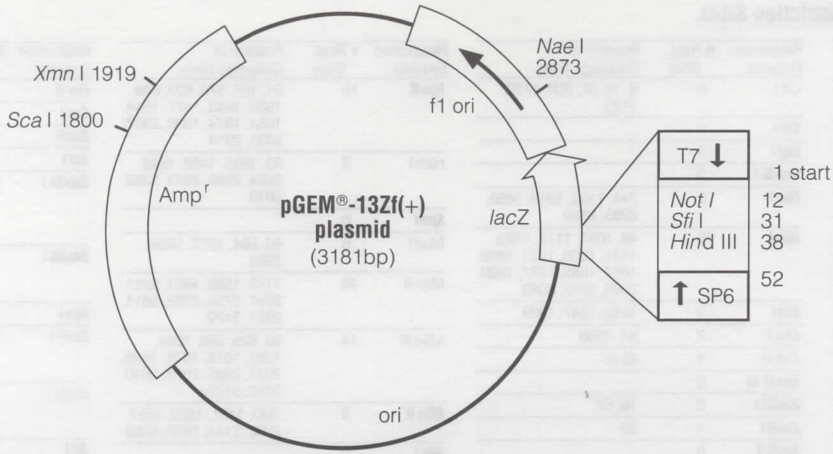


Figure 31. pGEM-13Zf(+) vector circle map.

Notes:

1. Sequence reference points:
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 52
   c. T7 RNA polymerase promoter 3165-3181
   d. SP6 RNA polymerase promoter 53-69
   e. multiple cloning sites 11-44
   f. *lacZ* start codon 90
   g. *lac* operon sequences 3002-3162; 76-323
   h. *lac* operator 110-126
   i. β-lactamase (Amp^r) coding region 1247-2107
   j. phage f1 region 2546-3001
   k. binding site of pUC/M13 forward sequencing primer 3122-3188
   l. binding site of pUC/M13 reverse sequencing primer 86-102
2. Specialized applications:
   a. in-frame expression of inserts subcloned from the λgt11 *Sfi-Not* vector
   b. ssDNA production
   c. blue/white screening for recombinants
   d. transcription *in vitro* from dual opposed promoters
3. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pGEM-13Zf(+) vector.

T7 Transcription Start

5′ . . . TGTAA TACGA CTCAC TATAG GGCGA AATTG

T7 Promoter

SP6 Transcription Start

GCGGC CGCTC CCTGG CCGAC TTGGC CGAAG CTTGA GTATT CTATA GTGTC ACCTA AATAG . . . 3′

Not I *Sfi* I *Hind* III SP6 Promoter

Figure 32. pGEM-13Zf(+) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase. The strand shown is complementary to the ssDNA strand produced by this vector.

Table 18. pGEM-13Zf(+) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2242
Acc I	0	
Acc III	0	
Acy I	2	1857, 2239
Afl II	0	
Aha II	2	1857, 2239
Alu I	19	40, 70, 92, 187, 251, 369, 595, 685, 731, 988, 1509, 1609, 1672, 2351, 2370, 2600, 2818, 2857, 3071
Alw44 I	3	741, 1987, 2484
Apa I	0	
Asu I	8	33, 1362, 1441, 1458, 1680, 2296, 2761, 3049
Ava I	0	
Ava II	2	1458, 1680
Bal I	0	
BamH I	0	
Ban I	3	171, 1268, 2807
Ban II	1	2845
Bbu I	0	
Bbv I	13	238, 319, 337, 756, 846, 849, 1055, 1358, 1749, 2360, 2936, 3004, 3077
Bgl I	3	31, 1440, 3014
Bgl II	0	
Bsm I	0	
Bsp1286 I	5	745, 1906, 1991, 2488, 2845
BssH II	0	
BstE II	0	
BstN I	6	22, 167, 455, 576, 589, 3118
Bsu36 I	0	
Cfo I	22	211, 276, 304, 337, 607, 674, 774, 948, 1057, 1450, 1543, 1830, 2212, 2312, 2415, 2920, 2928, 2954, 2976, 2985, 2998, 3022
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	6	702, 1111, 1277, 1817, 2243, 2478
Dra I	3	1186, 1205, 1897
Dra II	1	2296
Dra III	1	2770
Eco47 III	0	
Eco52 I	1	12
EcoR I	0	
EcoR V	0	
Fnu4H I	23	12, 15, 252, 333, 351, 354, 472, 627, 770, 835, 838, 1044, 1372, 1711, 1738, 2062, 2349, 2458, 2950, 2972, 2986, 3018, 3091
Fok I	5	1286, 1467, 1754, 2397, 3097,
Hae I	3	442, 453, 905
Hae II	4	305, 675, 2921, 2929
Hae III	16	14, 25, 34, 268, 442, 453, 471, 905, 1363, 1443, 1710, 2297, 2620, 2762, 3051, 3153
Hga I	5	538, 1116, 1846, 2404, 2987
HgiA I	4	745, 1906, 1991, 2488
Hha I	22	211, 276, 304, 337, 607, 674, 774, 948, 1057, 1450, 1543, 1830, 2212, 2312, 2415, 2920, 2928, 2954, 2976, 2985, 2998, 3022
Hinc II	0	
Hind III	1	38
Hinf I	8	262, 327, 402, 798, 1315, 2697, 2719, 3171
Hpa I	0	
Hpa II	14	145, 634, 781, 807, 997, 1401, 1435, 1502, 1612, 1832, 1854, 2355, 2389, 2872
Hph I	9	51, 1163, 1390, 1806, 2012, 2047, 2331, 2340, 2771
Kpn I	0	
Mae I	4	922, 1175, 1510, 2921
Mae II	10	1130, 1546, 1919, 2239, 2555, 2714, 2726, 2769, 2879, 3137
Mae III	14	57, 783, 846, 962, 1245, 1576, 1634, 1787, 1975, 2363, 2943, 2955, 3110, 3130
Mbo II	8	298, 1089, 1160, 1915, 1993, 2102, 2910, 3046
Mlu I	0	
Mnl I	13	275, 326, 534, 609, 858, 1258, 1329, 1487, 1693, 2286, 2346, 2797, 3062
Msp I	14	145, 634, 781, 807, 997, 1401, 1435, 1502, 1612, 1832, 1854, 2355, 2389, 2872
Mst I	2	1542, 3021
Nae I	1	2873
Nar I	0	
Nci I	6	807, 1503, 1832, 1854, 2355, 2390
Nco I	0	
Nde I	1	2491
Nhe I	0	
Nla III	10	85, 431, 1151, 1642, 1652, 1730, 1766, 2159, 2264, 2348
Nla IV	11	173, 459, 498, 1270, 1364, 1405, 1616, 2206, 2809, 2830, 2842
Not I	1	12
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	2	1690, 3042
Rsa I	2	1800, 2476
Rsr II	0	
Sac I	0	
Sac II	0	
Sal I	0	
Sau3A I	14	993, 1068, 1079, 1087, 1165, 1177, 1282, 1623, 1641, 1687, 1945, 1962, 1998, 3039
Sau96 I	8	33, 1362, 1441, 1458, 1680, 2296, 2761, 3049
Sca I	1	1800
ScrF I	12	22, 167, 455, 576, 589, 807, 1503, 1832, 1854, 2355, 2390, 3118
SfaN I	8	524, 1576, 1767, 2016, 2375, 2469, 2505, 2545
Sfi I	1	31
Sin I	2	1458, 1680
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	0	
Spo I	0	
Ssp I	3	2124, 2562, 2586
Sst I	0	
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	4	490, 527, 1971, 2803
Tth111 II	3	1017, 1024, 1056
Xba I	0	
Xho I	0	
Xho II	6	1068, 1079, 1165, 1171, 1945, 1962
Xma I	0	
Xma III	1	12
Xmn I	1	1919

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP70

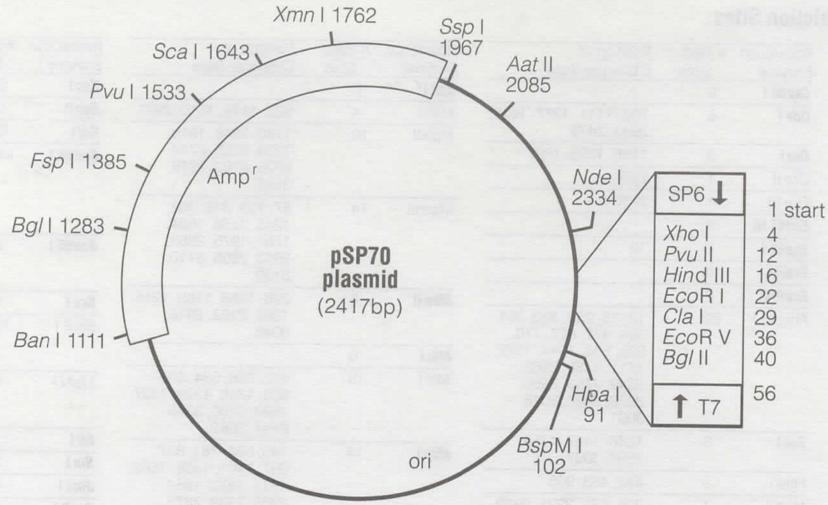


Figure 33. pSP70 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 56
   c. SP6 RNA polymerase promoter 2400-2417
   d. T7 RNA polymerase promoter 57-73
   e. multiple cloning sites 4-45
   f. β-lactamase (Amp^r) coding region 1090-1950
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pSP70 and pSP71 are identical except for the orientation of the multiple cloning site region.

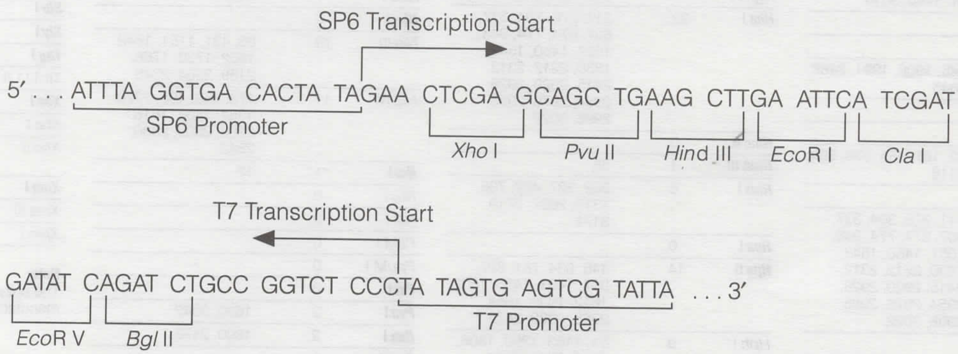


Figure 34. pSP70 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 19. pSP70 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2085
Acc I	0	
Acc III	0	
Acy I	2	1700, 2082
Afl II	0	
Aha II	2	1700, 2082
Alu I	11	12, 18, 212, 438, 574, 831, 1352, 1452, 1515, 2194, 2213
Alw44 I	3	584, 1830, 2327
Apa I	0	
Asu I	5	1205, 1284, 1301, 1523, 2139
Asu II	0	See **Csp45 I**
Ava I	1	4
Ava II	2	1301, 1523
Bal I	0	
BamH I	0	
Ban I	1	1111
Ban II	0	
Ban III	1	29
Bbu I	0	
Bbv I	10	21, 162, 180, 599, 689, 692, 898, 1201, 1592, 2203
Bcl I	0	
Bgl I	1	1283
Bgl II	1	40
Bsm I	0	
Bsp1286 I	4	588, 1749, 1834, 2331
BspM I	1	102
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	84, 296, 417, 430
BstX I	0	
Bsu36 I	0	
Cfo I	14	119, 147, 180, 450, 517, 617, 791, 900, 1293, 1386, 1723, 2055, 2155, 2258
Cfr I	2	109, 1551
Cla I	1	29
Csp I	0	
Csp45 I	0	
Dde I	6	545, 954, 1120, 1660, 2086, 2321
Dpn I	14	42, 838, 913, 924, 932, 1010, 1022, 1127, 1468, 1486, 1532, 1790, 1807, 1843
Dra I	3	1029, 1048, 1740
Dra II	1	2139
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	1	22
EcoR V	1	36
Fnu4H I	18	10, 176, 194, 197, 315, 470, 613, 678, 681, 887, 1215, 1554, 1581, 1676, 1905, 2192, 2301, 2359
Fok I	4	1129, 1310, 1597, 2240
Fsp I	1	1385
Hae I	3	285, 296, 748
Hae II	2	148, 518
Hae III	9	111, 285, 296, 314, 748, 1206, 1286, 1553, 2140
Hga I	4	381, 959, 1689, 2247
HgiA I	4	588, 1749, 1834, 2331
Hha I	14	119, 147, 180, 450, 517, 617, 791, 900, 1293, 1386, 1723, 2055, 2155, 2258
Hinc II	1	91
Hind II	1	91
Hind III	1	16
Hinf I	6	63, 105, 170, 245, 641, 1158
Hpa I	1	91
Hpa II	12	47, 477, 624, 650, 840, 1244, 1278, 1345, 1455, 1697, 2198, 2232
Hph I	7	1006, 1233, 1649, 1855, 1890, 2174, 2183
Kpn I	0	
Mae I	3	765, 1018, 1353
Mae II	4	973, 1389, 1762, 2082
Mae III	10	626, 689, 805, 1088, 1419, 1477, 1630, 1818, 2206, 2406
Mbo I	14	40, 836, 911, 922, 930, 1008, 1020, 1125, 1466, 1484, 1530, 1788, 1805, 1841
Mbo II	6	141, 932, 1003, 1758, 1836, 1945
Mlu I	0	
Mnl I	11	118, 169, 377, 452, 701, 1101, 1182, 1330, 1536, 2129, 2189
Msp I	12	47, 477, 624, 650, 840, 1244, 1278, 1345, 1455, 1697, 2198, 2232
Mst I	1	1385
Nae I	0	
Nar I	0	
Nci I	5	650, 1346, 1697, 2198, 2233
Nco I	0	
Nde I	1	2334
Nhe I	0	
Nla III	9	274, 994, 1485, 1495, 1573, 1609, 2002, 2107, 2191
Nla IV	7	302, 341, 1113, 1207, 1248, 1459, 2049
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	1	1533
Pvu II	1	12
Rsa I	2	1643, 2319
Rsr II	0	
Sac I	0	
Sac II	0	
Sal I	0	
Sau3A I	14	40, 836, 911, 922, 930, 1008, 1020, 1125, 1466, 1484, 1530, 1788, 1805, 1841
Sau96 I	5	1205, 1284, 1301, 1523, 2139
Sca I	1	1643
ScrF I	9	86, 298, 419, 432, 650, 1346, 1697, 2198, 2233
SfaN I	6	367, 1419, 1610, 1859, 2218, 2312
Sfi I	0	
Sin I	2	1301, 1523
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	0	
Spo I	0	
Ssp I	1	1967
Sst I	0	
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	5	5, 29, 77, 370, 1814
Tha I	11	117, 119, 317, 898, 1228, 1721, 2053, 2153, 2155, 2258, 2358
Tth111 II	3	860, 867, 899
Xba I	0	
Xho I	1	4
Xho II	7	40, 911, 922, 1008, 1020, 1788, 1895
Xma I	0	
Xma III	0	
Xmn I	1	1762

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP71

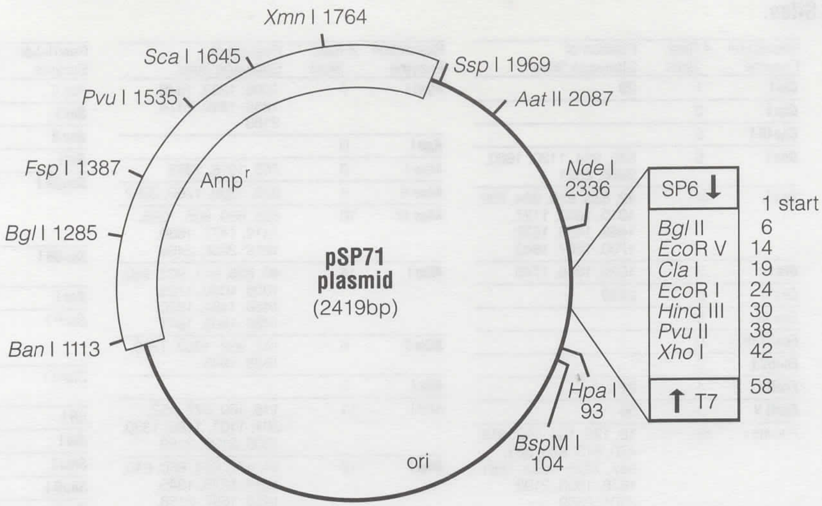


Figure 35. pSP71 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 58
   c. SP6 RNA polymerase promoter 2403-2419
   d. T7 RNA polymerase promoter 59-75
   e. multiple cloning sites 6-47
   f. β-lactamase (Amp^r) coding region 1092-1952
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pSP70 and pSP71 vectors are identical except for the orientation of the multiple cloning site region.

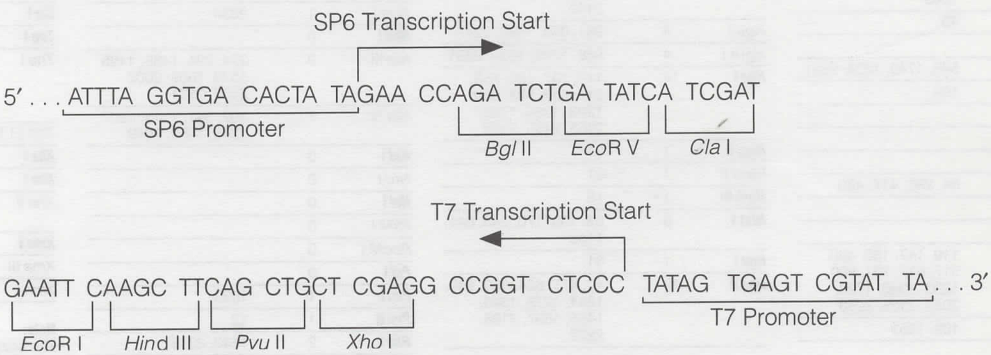


Figure 36. pSP71 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Promega

Table 20. pSP71 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2087
Acc I	0	
Acc III	0	
Acy I	2	1702, 2084
Afl II	0	
Aha II	2	1702, 2084
Alu I	11	32, 38, 214, 440, 576, 833, 1354, 1454, 1517, 2196, 2215
Alw44 I	3	586, 1832, 2329
Apa I	0	
Asu I	5	1207, 1286, 1303, 1525, 2141
Asu II	0	See **Csp45 I**
Ava I	1	42
Ava II	2	1303, 1525
Bal I	0	
BamH I	0	
Ban I	1	1113
Ban II	0	
Ban III	1	20
Bbu I	0	
Bbv I	10	25, 164, 182, 601, 691, 694, 900, 1203, 1594, 2205
Bcl I	0	
Bgl I	1	1285
Bgl II	1	6
Bsm I	0	
Bsp1286 I	4	590, 1751, 1836, 2333
BspM I	1	104
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	88, 300, 421, 434
BstX I	0	
Bsu36 I	0	
Cfo I	14	121, 149, 182, 452, 519, 619, 793, 902, 1295, 1388, 1725, 2057, 2157, 2260
Cfr I	2	111, 1553
Cla I	1	19
Csp I	0	
Csp45 I	0	
Dde I	6	547, 956, 1122, 1662, 2088, 2323
Dpn I	14	8, 840, 915, 926, 934, 1012, 1024, 1129, 1470, 1488, 1534, 1792, 1809, 1845
Dra I	3	1031, 1050, 1742
Dra II	1	2141
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	1	24
EcoR V	1	14
Fnu4H I	18	39, 178, 196, 199, 317, 472, 615, 680, 683, 889, 1217, 1556, 1583, 1678, 1907, 2194, 2303, 2361
Fok I	4	1131, 1312, 1599, 2242
Fsp I	1	1387
Hae I	3	287, 298, 750
Hae II	2	150, 520
Hae III	10	48, 113, 287, 298, 316, 750, 1208, 1288, 1555, 2142
Hga I	4	383, 961, 1691, 2249
HgiA I	4	590, 1751, 1836, 2333
Hha I	14	121, 149, 182, 452, 519, 619, 793, 902, 1295, 1388, 1725, 2057, 2157, 2260
Hinc II	1	93
Hind II	1	93
Hind III	1	30
Hinf I	6	65, 107, 172, 247, 643, 1160
Hpa I	1	93
Hpa II	12	49, 479, 626, 652, 842, 1246, 1280, 1347, 1457, 1699, 2200, 2234
Hph I	7	1008, 1235, 1651, 1857, 1892, 2176, 2185
Kpn I	0	
Mae I	3	767, 1020, 1355
Mae II	4	975, 1391, 1764, 2084
Mae III	10	628, 691, 807, 1090, 1421, 1479, 1632, 1820, 2208, 2408
Mbo I	14	6, 838, 913, 924, 932, 1010, 1022, 1127, 1468, 1486, 1532, 1790, 1807, 1843
Mbo II	6	143, 934, 1005, 1760, 1838, 1947
Mlu I	0	
Mnl I	12	37, 120, 171, 379, 454, 703, 1103, 1184, 1332, 1538, 2131, 2191
Msp I	12	49, 479, 626, 652, 842, 1246, 1280, 1347, 1457, 1699, 2200, 2234
Mst I	1	1387
Nae I	0	
Nar I	0	
Nci I	5	652, 1348, 1699, 2200, 2235
Nco I	0	
Nde I	1	2336
Nhe I	0	
Nla III	9	276, 996, 1487, 1497, 1575, 1611, 2004, 2109, 2193
Nla IV	7	304, 343, 1115, 1209, 1250, 1461, 2051
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	1	1535
Pvu II	1	38
Rsa I	2	1645, 2321
Rsr II	0	
Sac I	0	
Sac II	0	
Sal I	0	
Sau3A I	14	6, 838, 913, 924, 932, 1010, 1022, 1127, 1468, 1486, 1532, 1790, 1807, 1843
Sau96 I	5	1207, 1286, 1303, 1525, 2141
Sca I	1	1645
ScrF I	9	88, 300, 421, 434, 652, 1348, 1699, 2200, 2235
SfaN I	6	369, 1421, 1612, 1861, 2220, 2314
Sfi I	0	
Sin I	2	1303, 1525
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	0	
Spo I	0	
Ssp I	1	1969
Sst I	0	
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	5	19, 43, 79, 372, 1816
Tha I	11	119, 121, 319, 900, 1230, 1723, 2055, 2155, 2157, 2260, 2360
Tth111 II	3	862, 869, 901
Xba I	0	
Xho I	1	42
Xho II	7	6, 913, 924, 1010, 1022, 1790, 1897
Xma I	0	
Xma III	0	
Xmn I	1	1764

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP72

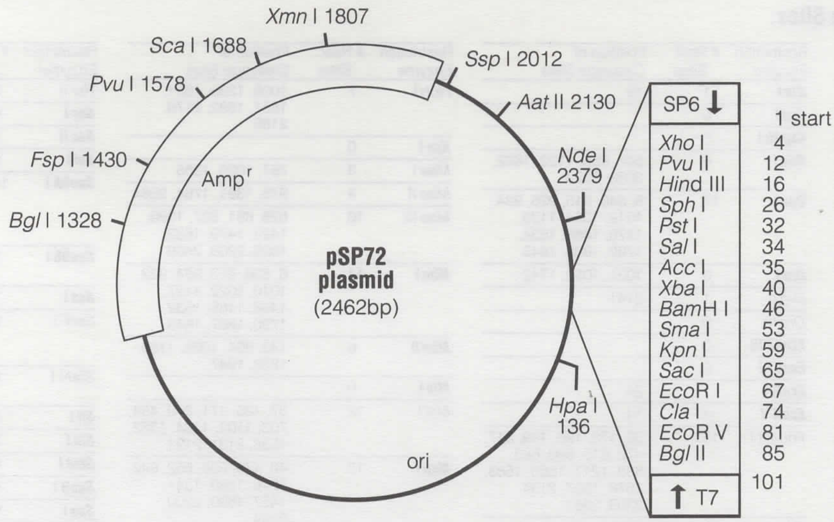


Figure 37. pSP72 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 101
   c. SP6 RNA polymerase promoter 2446-2462
   d. T7 RNA polymerase promoter 102-118
   e. multiple cloning sites 4-90
   f. β-lactamase (Amp^r) coding region 1135-1995
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pSP72 and pSP73 vectors are identical except for the orientation of the multiple cloning site region.

SP6 Transcription Start

5′ . . . ATTTA GGTGA CACTA TAGAA CTCGA GCAGC TGAAG CTTGC ATGCC TGCAG

SP6 Promoter — *Xho* I — *Pvu* II — *Hind* III — *Sph* I — *Pst* I

GTCGA CTCTA GAGGA TCCCC GGGTA CCGAG CTCGA ATTCA TCGAT

Sal I / *Acc* I — *Xba* I — *Bam*H I — *Sma* I — *Kpn* I — *Sac* I — *Eco*R I — *Cla* I

T7 Transcription Start

GATAT CAGAT CTGCC GGTCT CCCTA TAGTG AGTCG TATTA . . . 3′

*Eco*R V — *Bgl* II — T7 Promoter

Figure 38. pSP72 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 21. pSP72 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2130
Acc I	1	35
Acc III	0	
Acy I	2	1745, 2127
Afl II	0	
Aha II	2	1745, 2127
Alu I	12	12, 18, 63, 257, 483, 619, 876, 1397, 1497, 1560, 2239, 2258
Alw44 I	3	629, 1857, 2372
Apa I	0	
Asu I	5	1250, 1329, 1346, 1568, 2184
Asu II	0	See **Csp45 I**
Ava I	2	4, 51
Ava II	2	1346, 1568
Bal I	0	
BamH I	1	46
Ban I	2	55, 1156
Ban II	1	65
Ban III	1	75
Bbu I	1	26
Bbv I	10	21, 207, 225, 644, 734, 737, 943, 1246, 1637, 2248
Bcl I	0	
Bgl I	1	1328
Bgl II	1	85
Bsm I	0	
Bsp1286 I	5	65, 633, 1794, 1879, 2376
BspM I	2	21, 147
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	131, 343, 464, 477
BstX I	0	
Bsu36 I	0	
Cfo I	14	164, 192, 225, 495, 562, 662, 836, 945, 1338, 1431, 1768, 2100, 2200, 2303
Cfr I	2	154, 1596
Cla I	1	74
Csp I	0	
Csp45 I	0	
Dde I	6	590, 999, 1165, 1705, 2131, 2366
Dpn I	15	48, 87, 883, 958, 969, 977, 1055, 1067, 1172, 1513, 1531, 1577, 1835, 1852, 1888
Dra I	3	1074, 1093, 1785
Dra II	1	2184
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	1	67
EcoR V	1	81
Fnu4H I	18	10, 221, 239, 242, 360, 515, 658, 723, 726, 932, 1260, 1599, 1626, 1721, 1950, 2237, 2346, 2404
Fok I	4	1174, 1355, 1642, 2285
Fsp I	1	1430
Hae I	3	330, 341, 793
Hae II	2	193, 563
Hae III	9	156, 330, 341, 359, 793, 1251, 1331, 1598, 2185
Hga I	4	426, 1004, 1734, 2292
HgiA I	5	65, 633, 1794, 1879, 2376
Hha I	14	164, 192, 225, 495, 562, 662, 836, 945, 1338, 1431, 1768, 2100, 2200, 2303
Hinc II	2	36, 136
Hind II	2	36, 136
Hind III	1	16
Hinf I	7	37, 108, 150, 215, 290, 686, 1203
Hpa I	1	136
Hpa II	13	52, 92, 522, 669, 695, 885, 1289, 1323, 1390, 1500, 1742, 2243, 2277
Hph I	7	1051, 1278, 1694, 1900, 1935, 2219, 2228
Kpn I	1	59
Mae I	4	41, 810, 1063, 1398
Mae II	4	1018, 1434, 1807, 2127
Mae III	10	671, 734, 850, 1133, 1464, 1522, 1675, 1863, 2251, 2451
Mbo I	15	46, 85, 881, 956, 967, 975, 1053, 1065, 1170, 1511, 1529, 1575, 1833, 1850, 1886
Mbo II	6	186, 977, 1048, 1803, 1881, 1990
Mlu I	0	
Mnl I	12	36, 163, 214, 422, 497, 746, 1146, 1227, 1375, 1581, 2174, 2234
Msp I	13	52, 92, 522, 669, 695, 885, 1289, 1323, 1390, 1500, 1742, 2243, 2277
Mst I	1	1430
Nae I	0	
Nar I	0	
Nci I	7	52, 53, 695, 1391, 1742, 2243, 2278
Nco I	0	
Nde I	1	2379
Nhe I	0	
Nla III	10	26, 319, 1039, 1530, 1540, 1618, 1654, 2047, 2152, 2236
Nla IV	9	48, 57, 347, 386, 1158, 1252, 1293, 1504, 2094
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	1	32
Pvu I	1	1578
Pvu II	1	12
Rsa I	3	57, 1688, 2364
Rsr II	0	
Sac I	1	65
Sac II	0	
Sal I	1	34
Sau3A I	15	46, 85, 881, 956, 967, 975, 1053, 1065, 1170, 1511, 1529, 1575, 1833, 1850, 1886
Sau96 I	5	1250, 1329, 1346, 1568, 2184
Sca I	1	1688
ScrF I	11	52, 53, 131, 343, 464, 477, 695, 1391, 1742, 2243, 2278
SfaN I	6	412, 1464, 1655, 1904, 2263, 2357
Sfi I	0	
Sin I	2	1346, 1568
Sma I	1	53
SnaB I	0	
Spe I	0	
Sph I	1	26
Spo I	0	
Ssp I	1	2012
Sst I	1	65
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	7	5, 35, 65, 74, 122, 415, 1859
Tha I	11	162, 164, 362, 943, 1273, 1766, 2098, 2198, 2200, 2303, 2403
Tth111 II	3	905, 912, 944
Xba I	1	40
Xho I	1	4
Xho II	8	46, 85, 956, 967, 1053, 1065, 1833, 1850
Xma I	1	53
Xma III	0	
Xmn I	1	1807

Note:
The enzymes listed in boldface type aremanufactured by Promega.

Vector Maps

pSP73

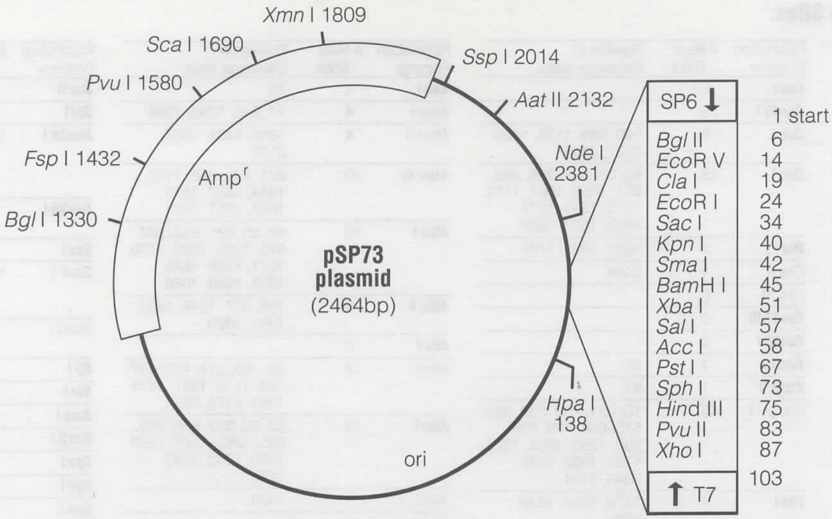


Figure 39. pSP73 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T7 RNA polymerase transcription initiation site 103
   c. SP6 RNA polymerase promoter 2448-2464
   d. T7 RNA polymerase promoter 104-120
   e. multiple cloning sites 6-92
   f. β-lactamase (Amp^r) coding region 1137-1997
2. Specialized application:
   a. transcription *in vitro* from dual opposed promoters
3. The pSP72 and pSP73 vectors are identical except for the orientation of the multiple cloning site regions.

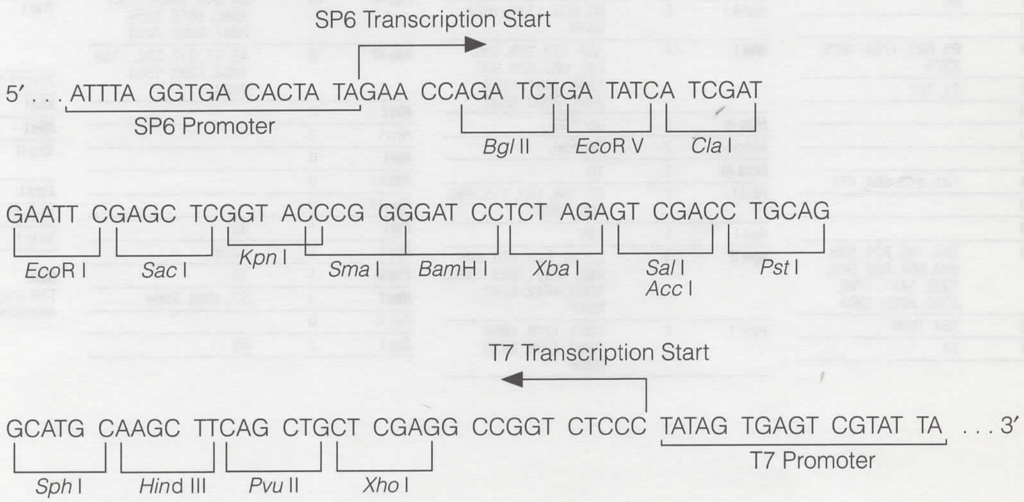


Figure 40. pSP73 plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase.

Table 22. pSP73 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2132
Acc I	1	58
Acc III	0	
Acy I	2	1747, 2129
Afl II	0	
Aha II	2	1747, 2129
Alu I	12	32, 77, 83, 259, 485, 621, 878, 1399, 1499, 1562, 2241, 2260
Alw 44 I	3	631, 1877, 2374
Apa I	0	
Asu I	5	1252, 1331, 1348, 1570, 2186
Asu II	0	See **Csp45 I**
Ava I	2	40, 87
Ava II	2	1348, 1570
Bal I	0	
BamH I	1	45
Ban I	2	36, 1158
Ban II	1	34
Ban III	1	20
Bbu I	1	73
Bbv I	10	70, 209, 227, 646, 736, 739, 945, 1248, 1639, 2250
Bcl I	0	
Bgl I	1	1330
Bgl II	1	6
Bsm I	0	
Bsp1286 I	5	34, 635, 1796, 1881, 2378
BspM I	2	70, 149
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	133, 345, 466, 479
BstX I	0	
Bsu 36 I	0	
Cfo I	14	166, 194, 227, 497, 564, 664, 838, 947, 1340, 1433, 1770, 2102, 2202, 2305
Cfr I	2	156, 1598
Cla I	1	19
Csp I	0	
Csp45 I	0	
Dde I	6	592, 1001, 1167, 1707, 2133, 2368
Dpn I	15	8, 47, 885, 960, 971, 979, 1057, 1069, 1174, 1515, 1533, 1579, 1837, 1854, 1890
Dra I	3	1076, 1095, 1787
Dra II	1	2186
Dra III	0	
Eco 47 III	0	
Eco 52 I	0	
EcoR I	1	24
EcoR V	1	14
Fnu4H I	18	84, 223, 241, 244, 362, 517, 660, 725, 728, 934, 1262, 1601, 1628, 1723, 1952, 2239, 2348, 2406
Fok I	4	1176, 1357, 1644, 2287
Fsp I	1	1432
Hae I	3	332, 343, 795
Hae II	2	195, 565
Hae III	10	93, 158, 332, 343, 361, 795, 1253, 1333, 1600, 2187
Hga I	4	428, 1006, 1736, 2294
HgiA I	5	34, 635, 1796, 1881, 2378
Hha I	14	166, 194, 227, 497, 564, 664, 838, 947, 1340, 1433, 1770, 2102, 2202, 2305
Hinc II	2	59, 138
Hind II	2	59, 138
Hind III	1	75
Hinf I	7	55, 110, 152, 217, 292, 688, 1205
Hpa I	1	138
Hpa II	13	41, 94, 524, 671, 697, 887, 1291, 1325, 1392, 1502, 1744, 2245, 2279
Hph I	7	1053, 1280, 1696, 1902, 1937, 2221, 2230
Kpn I	1	40
Mae I	4	52, 812, 1065, 1400
Mae II	4	1020, 1436, 1809, 2129
Mae III	10	673, 736, 852, 1135, 1466, 1524, 1677, 1865, 2253, 2453
Mbo I	15	6, 45, 883, 958, 969, 977, 1055, 1067, 1172, 1513, 1531, 1577, 1835, 1852, 1888
Mbo II	6	188, 979, 1050, 1805, 1883, 1992
Mlu I	0	
Mnl I	13	59, 82, 165, 216, 424, 499, 748, 1148, 1229, 1377, 1583, 2176, 2236
Msp I	13	41, 94, 524, 671, 697, 887, 1291, 1325, 1392, 1502, 1744, 2245, 2279
Mst I	1	1432
Nae I	0	
Nar I	0	
Nci I	7	41, 42, 697, 1393, 1744, 2245, 2280
Nco I	0	
Nde I	1	2381
Nhe I	0	
Nla III	10	73, 321, 1041, 1532, 1542, 1620, 1656, 2049, 2154, 2238
Nla IV	9	38, 47, 349, 388, 1160, 1254, 1295, 1506, 2096
Not I	0	
Nru I	0	
Nsi I	0	
PflM I	0	
PpuM I	0	
Pst I	1	67
Pvu I	1	1580
Pvu II	1	83
Rsa I	3	38, 1690, 2366
Rsr II	0	
Sac I	1	34
Sac II	0	
Sal I	1	57
Sau3A I	15	6, 45, 883, 958, 969, 977, 1055, 1067, 1172, 1513, 1531, 1577, 1835, 1852, 1888
Sau96 I	5	1252, 1331, 1348, 1570, 2186
Sca I	1	1690
ScrF I	11	41, 42, 133, 345, 466, 479, 697, 1393, 1744, 2245, 2280
SfaN I	6	414, 1466, 1657, 1906, 2265, 2359
Sfi I	0	
Sin I	2	1348, 1570
Sma I	1	42
SnaB I	0	
Spe I	0	
Sph I	1	73
Spo I	0	
Ssp I	1	2014
Sst I	1	34
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	7	19, 28, 88, 124, 417, 1861
Tha I	11	164, 166, 364, 945, 1275, 1768, 2100, 2200, 2202, 2305, 2405
Tth111 II	3	907, 914, 946
Xba I	1	51
Xho I	1	87
Xho II	8	6, 45, 958, 969, 1055, 1067, 1835, 1852
Xma I	1	42
Xma III	0	
Xmn I	1	1809

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP64

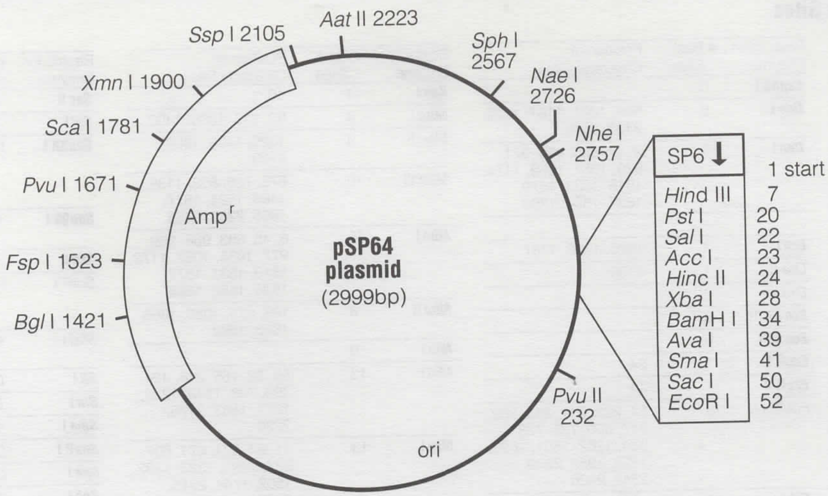


Figure 41. pSP64 vector circle map.

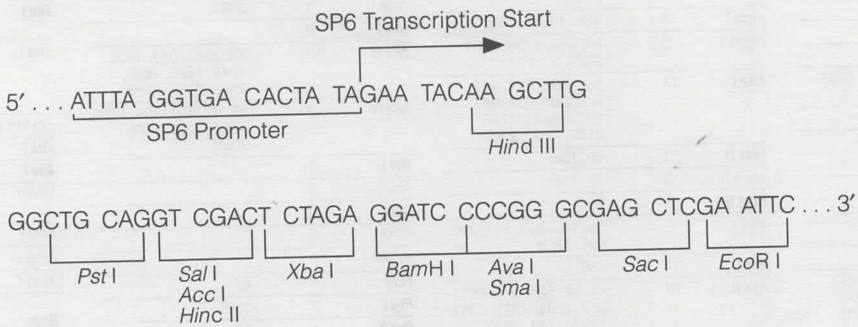


Figure 42. pSP64 plasmid promoter and multiple cloning site sequence. Base #1 is the transcription start site for SP6 RNA polymerase. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site — 1
   b. SP6 RNA polymerase promoter — 2983-2999
   c. multiple cloning sites — 7-57
   d. *lac*Z start codon — 71
   e. *lac* operon sequences — 57-286
   f. *lac* operator — 91-107
   g. β-lactamase (Amp^r) coding region — 1228-2088
2. Specialized application:
   a. transcription *in vitro* from the SP6 promoter
3. The pSP64 and pSP65 vectors are identical except for the orientation of the multiple cloning site region.

Reference:

1. Melton, D.A., *et al.* (1984) *Nucl. Acids Res.* **12**, 7035-7056.

Table 23. pSP64 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2223
Acc I	1	23
Acc III	0	
Acy I	5	1838, 2220, 2578, 2692, 2713
Afl II	0	
Aha II	5	1838, 2220, 2578, 2692, 2713
Alu I	14	9, 48, 73, 168, 232, 350, 576, 712, 969, 1490, 1590, 1653, 2332, 2351
Alw44 I	3	722, 1968, 2465
Apa I	0	
Asu I	6	1343, 1422, 1439, 1661, 2277, 2602
Ava I	1	39
Ava II	2	1439, 1661
Avr II	0	
Bal I	0	
BamH I	1	34
Ban I	5	152, 1249, 2577, 2691, 2712
Ban II	3	50, 2644, 2658
Bbu I	1	2567
Bbv I	12	2, 219, 300, 318, 737, 827, 830, 1036, 1339, 1730, 2341, 2523
Bcl I	0	
Bgl I	1	1421
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	50, 726, 1887, 1972, 2469, 2542, 2644, 2658
BspM I	1	9
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	148, 436, 557, 570
BstX I	0	
Bsu 36 I	0	
Cfo I	19	192, 257, 285, 318, 588, 655, 755, 929, 1038, 1431, 1524, 1861, 2193, 2293, 2396, 2580, 2634, 2694, 2715
Cfr I	4	247, 1689, 2594, 2726
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	7	683, 1092, 1258, 1798, 2224, 2459, 2823
Dpn I	16	36, 976, 1051, 1062, 1070, 1148, 1160, 1265, 1606, 1624, 1670, 1928, 1945, 1981, 2661, 2752
Dra I	3	1167, 1186, 1878
Dra II	2	2277, 2602
Dra III	0	
Eco47 III	1	2633
Eco52 I	0	
EcoR I	1	52
EcoR V	0	
Fnu4H I	22	16, 233, 314, 332, 335, 453, 608, 751, 816, 819, 1025, 1353, 1692, 1719, 1814, 2043, 2330, 2439, 2512, 2546, 2549, 2941
Fok I	4	1267, 1448, 1735, 2378
Fsp I	1	1523
Hae II	6	286, 656, 2581, 2635, 2695, 2716
Hae III	13	249, 423, 434, 452, 886, 1344, 1424, 1691, 2278, 2532, 2596, 2604, 2728
Hga I	6	519, 1097, 1827, 2385, 2486, 2725
HgiA I	6	50, 726, 1887, 1972, 2469, 2542
Hha I	19	192, 257, 285, 318, 588, 655, 755, 929, 1038, 1431, 1524, 1861, 2193, 2293, 2396, 2580, 2634, 2694, 2715
Hinc II	1	24
Hind II	1	24
Hind III	1	7
Hinf I	8	25, 243, 308, 383, 779, 1296, 2494, 2848
Hpa I	0	
Hpa II	18	40, 126, 615, 762, 788, 978, 1382, 1416, 1483, 1593, 1835, 2336, 2370, 2593, 2716, 2725, 2740, 2795
Hph I	10	1, 144, 1371, 1787, 1993, 2028, 2313, 2321, 2685, 2730
Kpn I	0	
Mae I	5	29, 903, 1156, 1491, 2758
Mae II	4	1111, 1527, 1900, 2220
Mae III	11	764, 827, 943, 1226, 1557, 1615, 1768, 1956, 2344, 2811, 2988
Mbo I	16	34, 974, 1049, 1060, 1068, 1146, 1158, 1263, 1604, 1622, 1668, 1926, 1943, 1979, 2659, 2750
Mbo II	7	279, 1070, 1141, 1896, 1974, 2083, 2654
Mlu I	0	
Mnl I	14	24, 256, 307, 515, 590, 839, 1239, 1320, 1468, 1674, 2267, 2327, 2521, 2740
Msp I	18	40, 126, 615, 762, 788, 578, 1382, 1416, 1483, 1593, 1835, 2336, 2370, 2593, 2716, 2725, 2740, 2795
Mst I	1	1523
Nae I	1	2726
Nar I	3	2578, 2692, 2713
Nci I	9	40, 41, 788, 1484, 1835, 2336, 2371, 2593, 2796
Nco I	0	
Nde I	0	
Nhe I	1	2757
Nla III	12	66, 412, 1132, 1623, 1633, 1711, 1747, 2140, 2245, 2329, 2567, 2640
Nla IV	13	36, 154, 440, 479, 1251, 1345, 1386, 1597, 2187, 2579, 2603, 2693, 2714
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	20
Pvu I	1	1671
Pvu II	1	232
Rsa I	3	1781, 2457, 2921
Rsr II	0	
Sac I	1	50
Sac II	0	
Sal I	1	22
Sau3A I	16	34, 974, 1049, 1060, 1068, 1146, 1158, 1263, 1604, 1622, 1668, 1926, 1943, 1979, 2659, 2750
Sau96 I	6	1343, 1422, 1439, 1661, 2277, 2602
Sca I	1	1781
ScrF I	13	40, 41, 148, 436, 557, 570, 788, 1484, 1835, 2336, 2371, 2593, 2796
SfaN I	9	505, 1557, 1748, 1997, 2356, 2450, 2711, 2723, 2890
Sfi I	0	
Sin I	2	1439, 1661
Sma I	1	41
SnaB I	0	
Spe I	0	
Sph I	1	2567
Spo I	0	
Ssp I	1	2105
Sst I	1	50
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	5	23, 50, 508, 1952, 2475
Tha I	11	255, 257, 455, 1036, 1366, 1859, 2191, 2291, 2293, 2396, 2940
Tth111 II	3	998, 1005, 1037
Xba I	1	28
Xho I	0	
Xho II	8	34, 1049, 1060, 1146, 1158, 1926, 1943, 2750
Xma I	1	39
Xma III	0	
Xmn I	1	1900

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP65

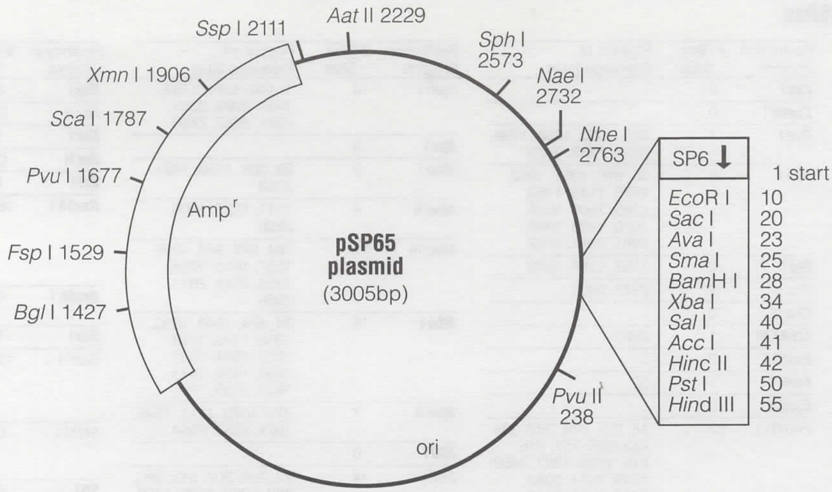


Figure 43. pSP65 vector circle map.

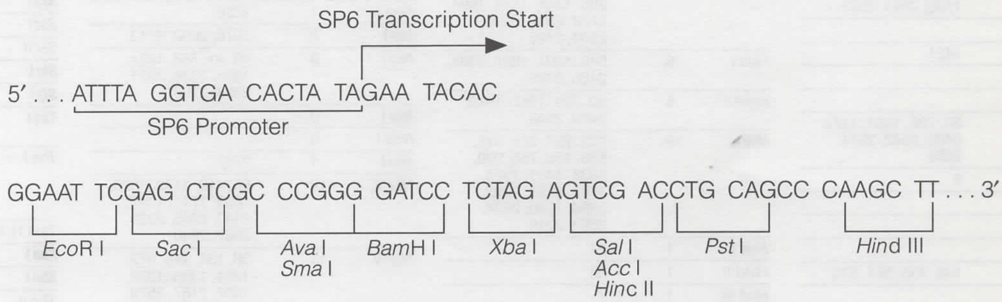


Figure 44. pSP65 plasmid promoter and multiple cloning site sequence. Base #1 is the transcription start site for SP6 RNA polymerase. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase promoter 2989-3005
   c. multiple cloning sites 10-60
   d. *lacZ* start codon 77
   e. *lac* operon sequences 63-292
   f. *lac* operator 97-113
   g. β-lactamase (Amp^r) coding region 1234-2094
2. Specialized application:
   a. transcription *in vitro* from the SP6 promoter
3. The pSP64 and pSP65 vectors are identical except for the orientation of the multiple cloning site region.

Reference:

1. Melton, D.A., *et al.* (1984) *Nucl. Acids Res.* **12**, 7035-7056.

Table 24. pSP65 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2229
Acc I	1	41
Acc III	0	
Acy I	5	1844, 2226, 2584, 2698, 2719
Afl II	0	
Aha II	5	1844, 2226, 2584, 2698, 2719
Alu I	14	18, 57, 79, 174, 238, 356, 582, 718, 975, 1496, 1596, 1659, 2338, 2357
Alw44 I	3	728, 1974, 2471
Apa I	0	
Asu I	6	1349, 1428, 1445, 1667, 2283, 2608
Asu II	0	
Ava I	1	23
Ava II	2	1445, 1667
Avr II	0	
Bal I	0	
BamH I	1	28
Ban I	5	158, 1255, 2583, 2697, 2718
Ban II	3	20, 2650, 2664
Bbu I	1	2573
Bbv I	12	60, 225, 306, 324, 743, 833, 836, 1042, 1345, 1736, 2347, 2529
Bcl I	0	
Bgl I	1	1427
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	20, 732, 1893, 1978, 2475, 2548, 2650, 2664
BspM I	1	53
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	154, 442, 563, 576
BstX I	0	
Bsu36 I	0	
Cfo I	19	198, 263, 291, 324, 594, 661, 761, 935, 1044, 1437, 1530, 1867, 2199, 2299, 2402, 2586, 2640, 2700, 2721
Cfr I	4	253, 1695, 2599, 2732
Csp I	0	
Csp45 I	0	
Dde I	7	689, 1098, 1264, 1804, 2230, 2465, 2829
Dpn I	16	30, 982, 1057, 1068, 1076, 1154, 1166, 1291, 1612, 1630, 1676, 1934, 1951, 1987, 2667, 2758
Dra I	3	1173, 1192, 1884
Dra II	2	2283, 2608
Dra III	0	
Eco47 III	1	2639
Eco52 I	0	
EcoR I	1	10
Fnu4H I	22	49, 239, 320, 338, 341, 459, 614, 757, 822, 825, 1031, 1359, 1698, 1725, 1820, 2049, 2336, 2445, 2518, 2552, 2555, 2947
Fok I	4	1273, 1454, 1741, 2384
Fsp I	1	1529
Hae II	6	292, 662, 2587, 2641, 2701, 2722
Hae III	13	255, 429, 440, 458, 892, 1350, 1430, 1697, 2284, 2538, 2602, 2610, 2734
Hga I	6	525, 1103, 1833, 2391, 2942, 2731
HgiA I	6	20, 732, 1893, 1978, 2475, 2548
Hha I	19	198, 263, 291, 324, 594, 661, 761, 935, 1044, 1437, 1530, 1867, 2199, 2299, 2402, 2586, 2640, 2700, 2721
Hinc II	1	42
Hind II	1	42
Hind III	1	55
Hinf I	8	38, 249, 314, 389, 785, 1302, 2500, 2854
Hpa I	0	
Hpa II	18	24, 132, 621, 768, 794, 984, 1388, 1422, 1489, 1599, 1841, 2342, 2376, 2599, 2722, 2731, 2746, 2801
Hph I	10	1, 1150, 1377, 1793, 1999, 2034, 2318, 2327, 2691, 2736
Kpn I	0	
Mae I	5	35, 909, 1162, 1497, 2764
Mae II	4	1117, 1533, 1906, 2226
Mae III	11	770, 833, 949, 1232, 1563, 1621, 1774, 1962, 2350, 2817, 2994
Mbo I	16	28, 980, 1055, 1066, 1074, 1152, 1164, 1269, 1610, 1628, 1674, 1932, 1949, 1985, 2665, 2756
Mbo II	7	285, 1076, 1147, 1902, 1980, 2089, 2660
Mlu I	0	
Mnl I	14	42, 262, 313, 521, 596, 845, 1245, 1326, 1474, 1680, 2273, 2333, 2527, 2746
Msp I	18	46, 132, 621, 768, 794, 984, 1388, 1422, 1489, 1599, 1841, 2342, 2376, 2599, 2722, 2731, 2746, 2801
Msp I	18	46, 132, 621, 768, 794, 984, 1388, 1422, 1489, 1599, 1841, 2342, 2376, 2599, 2722, 2731, 2746, 2801
Mst I	1	1529
Nae I	1	2732
Nar I	3	2584, 2698, 2719
Nci I	9	24, 25, 794, 1490, 1841, 2342, 2377, 2599, 2802
Nco I	0	
Nde I	0	
Nhe I	1	2763
Nla III	12	72, 418, 1138, 1629, 1639, 1717, 1753, 2146, 2251, 2335, 2573, 2646
Nla IV	13	30, 160, 446, 485, 1257, 1351, 1392, 1603, 2193, 2585, 2609, 2699, 2720
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	50
Pvu I	1	1677
Pvu II	1	238
Rsa I	3	1787, 2463, 2927
Rsr II	0	
Sac I	1	20
Sac II	0	
Sal I	1	40
Sau3A I	16	28, 980, 1055, 1066, 1074, 1152, 1164, 1269, 1610, 1628, 1674, 1931, 1949, 1985, 2665, 2756
Sau96 I	6	1349, 1428, 1445, 1667, 2283, 2608
Sca I	1	1787
ScrF I	13	24, 25, 154, 442, 563, 576, 794, 1490, 1841, 2342, 2377, 2599, 2802
SfaN I	9	511, 1563, 1754, 2003, 2362, 2456, 2717, 2729, 2896
Sfi I	0	
Sin I	2	1445, 1667
Sma I	1	25
SnaB I	0	
Spe I	0	
Sph I	1	2573
Spo I	0	
Ssp I	1	2111
Sst I	1	20
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	5	14, 41, 514, 1958, 2481
Tha I	11	261, 263, 461, 1042, 1372, 1865, 2197, 2297, 2299, 2402, 2946
Tth111 II	3	1004, 1011, 1043
Xba I	1	34
Xho I	0	
Xho II	8	28, 1055, 1066, 1152, 1164, 1932, 1949, 2756
Xma I	1	23
Xma III	0	
Xmn I	1	1906

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSP64(polyA)

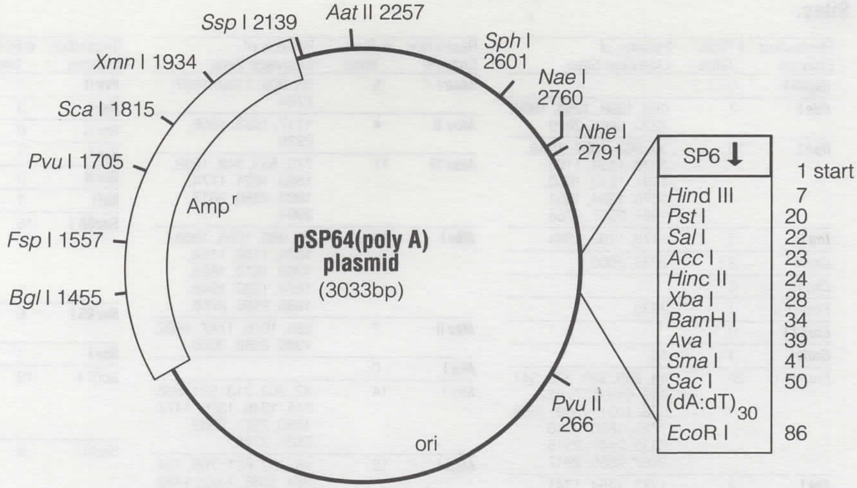


Figure 45. pSP64(polyA) vector circle map.

Notes:

1. Sequence reference points:
   - a. SP6 RNA polymerase transcription initiation site 1
   - b. SP6 RNA polymerase promoter 3017-3033
   - c. multiple cloning sites 7-91
   - d. *lac*Z start codon 105
   - e. *lac* operon sequences 91-320
   - f. *lac* operator 125-141
   - g. β-lactamase (Amp^r) coding region 1262-2122
2. Specialized application:
   - a. synthesis *in vitro* of (polyA)+ mRNA

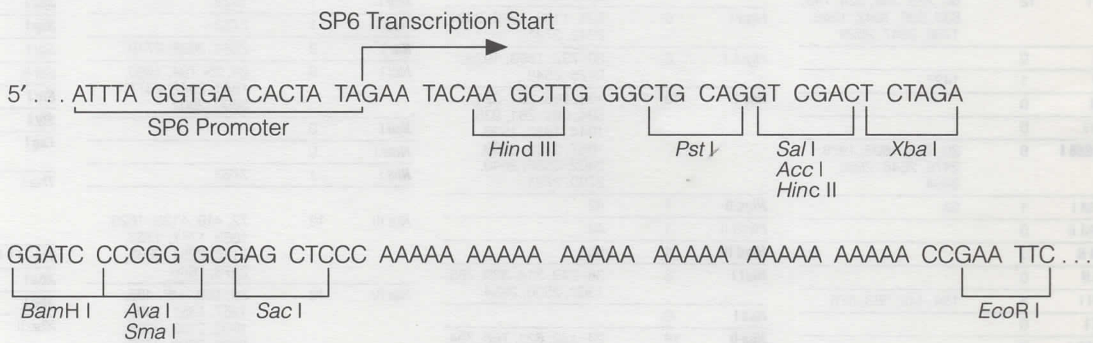


Figure 46.pSP64 (polyA) plasmid promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase.

Table 25. pSP64(polyA) Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	2257
Acc I	1	23
Acc III	0	
Acy I	5	1872, 2254, 2612, 2726, 2747
Afl II	0	
Aha II	5	1872, 2254, 2612, 2726, 2747
Alu I	14	9, 48, 107, 202, 266, 384, 610, 700, 746, 1003, 1524, 1624, 1687, 2366, 2385
Alw44 I	3	756, 2002, 2499
Apa I	0	
Asu I	6	1377, 1456, 1473, 1695, 2311, 2636
Ava I	1	39
Ava II	2	1473, 1695
Avr II	0	
Bal I	0	
BamH I	1	34
Ban I	5	186, 1283, 2611, 2725, 2746
Ban II	3	50, 2678, 2692
Bbu I	1	2601
Bbv I	12	2, 253, 334, 352, 771, 861, 864, 1070, 1373, 1764, 2375, 2557
Bcl I	0	
Bgl I	1	1455
Bgl II	0	
Bsm I	0	
Bsp1286 I	8	50, 760, 1921, 2006, 2503, 2576, 2678, 2692
BspM I	1	9
BspM II	0	
BssH II	0	
BstE II	0	
BstN I	4	182, 470, 591, 604
BstX I	0	
Bsu36 I	0	
Cfo I	19	226, 291, 319, 352, 622, 689, 789, 963, 1072, 1465, 1558, 1895, 2227, 2327, 2430, 2614, 2668, 2728, 2749
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	7	717, 1126, 1292, 1832, 2258, 2493, 2857
Dpn I	16	36, 1010, 1085, 1096, 1104, 1182, 1194, 1299, 1640, 1658, 1704, 1962, 1979, 2015, 2695, 2786
Dra I	3	1201, 1220, 1912
Dra II	2	2311, 2636
Dra III	0	
Eco47 III	1	2667
Eco52 I	0	
EcoR I	1	86
EcoR V	0	
Fnu4H I	22	16, 267, 348, 366, 369, 487, 642, 785, 850, 853, 1059, 1387, 1726, 1753, 1848, 2077, 2364, 2473, 2546, 2580, 2583, 2975
Fok I	4	1301, 1482, 1769, 2412
Fsp I	1	1557
Hae II	6	320, 690, 2615, 2669, 2729, 2750
Hae III	13	283, 457, 468, 486, 920, 1378, 1458, 1725, 2312, 2566, 2630, 2638, 2762
Hga I	6	553, 1131, 1861, 2419, 2520, 2759
HgiA I	6	50, 760, 1921, 2006, 2503, 2576
Hha I	19	226, 291, 319, 352, 622, 689, 789, 963, 1072, 1465, 1558, 1895, 2227, 2327, 2430, 2614, 2668, 2728, 2749
Hinc II	1	24
Hind II	1	24
Hind III	1	7
Hinf I	8	25, 277, 342, 417, 813, 1330, 2528, 2882
Hpa I	0	
Hpa II	18	40, 160, 649, 796, 822, 1012, 1416, 1450, 1517, 1627, 1869, 2370, 2404, 2627, 2750, 2759, 2774, 2829
Hph I	9	1178, 1405, 1821, 2027, 2062, 2346, 2355, 2719, 2764
Kpn I	0	
Mae I	5	29, 937, 1190, 1525, 2792
Mae II	4	1145, 1561, 1934, 2254
Mae III	11	798, 861, 977, 1260, 1591, 1649, 1802, 1990, 2378, 2845, 3022
Mbo I	16	34, 1008, 1083, 1094, 1102, 1180, 1192, 1297, 1638, 1656, 1702, 1960, 1977, 2013, 2693, 2784
Mbo II	7	313, 1104, 1175, 1930, 2008, 2117, 2688
Mlu I	0	
Mnl I	14	24, 290, 341, 549, 624, 873, 1273, 1354, 1502, 1708, 2301, 2361, 2555, 2774
Msp I	18	40, 160, 649, 796, 822, 1012, 1416, 1450, 1517, 1627, 1869, 2370, 2404, 2627, 2750, 2759, 2774, 2829
Mst I	1	1557
Nae I	1	2760
Nar I	3	2612, 2726, 2747
Nci I	9	40, 41, 822, 1518, 1869, 2370, 2405, 2627, 2830
Nco I	0	
Nde I	0	
Nhe I	1	2791
Nla III	12	100, 446, 1166, 1657, 1667, 1745, 1781, 2174, 2279, 2363, 2601, 2674
Nla IV	13	36, 188, 474, 513, 1285, 1379, 1420, 1631, 2221, 2613, 2637, 2727, 2748
Not I	0	
Nru I	0	
Nsi I	0	
PpuM I	0	
Pst I	1	20
Pvu I	1	1705
Pvu II	1	266
Rsa I	3	1815, 2491, 2955
Rsr II	0	
Sac I	1	50
Sac II	0	
Sal I	1	22
Sau3A I	16	34, 1008, 1083, 1094, 1102, 1180, 1192, 1297, 1638, 1656, 1702, 1960, 1977, 2013, 2693, 2784
Sau96 I	6	1377, 1456, 1473, 1695, 2311, 2636
Sca I	1	1815
ScrF I	13	40, 41, 182, 470, 591, 604, 822, 1518, 1869, 2370, 2405, 2627, 2830
SfaN I	9	539, 1591, 1782, 2031, 2390, 2484, 2745, 2757, 2924
Sfi I	0	
Sin I	2	1473, 1695
Sma I	1	41
Sph I	1	2601
Spo I	0	
Ssp I	1	2139
Sst I	1	50
Sst II	0	
Stu I	0	
Sty I	0	
Taq I	4	23, 542, 1986, 2509
Tha I	11	289, 291, 489, 1070, 1400, 1893, 2225, 2325, 2327, 2430, 2974
Tth111 II	3	1032, 1039, 1071
Xba I	1	28
Xho II	8	34, 1083, 1094, 1180, 1192, 1960, 1977, 2784
Xma I	1	39
Xma III	0	
Xmn I	1	1934

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pBR322

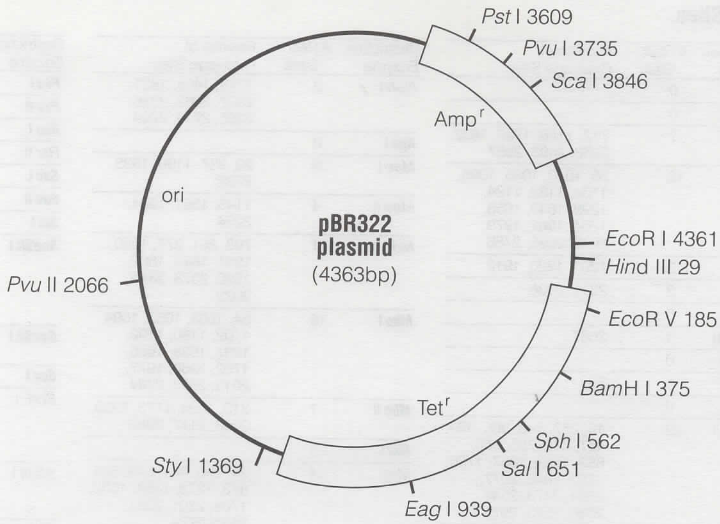


Figure 47. pBR322 vector circle map.

pBR322

Table 26. Enzymes With Single Recognition Sites in pBR322.

Aat II	*Afl* III	*Ava* I	*Bal* I
*Bam*H I	*Ban* I	*Cfa* I	*Eco*R I
*Eco*R V	*Hin*d III	*Nde* I	*Nhe* I
Nru I	*Pst* I	*Pvu* I	*Pvu* II
Sal I	*Sca* I	*Sna* I	*Sph* I
Ssp I	*Sty* I	*Tth*111 I	*Xma* III

Table 27. Enzymes Which Do Not Cut pBR322

Afl II	*Apa* I	*Avr* II	*Bcl* I
Bgl II	*Bss*H I	*Bst*E II	*Bst*X I
*Csp*45 I	*Dra* III	*Esp* I	*Hpa* I
Kpn I	*Mlu* I	*Mst* II	*Nco* I
Not I	*Nsi* I	*Rsr* II	*Sac* I
Sac II	*Sfi* I	*Sma* I	*Sna*B I
Spe I	*Stu* I	*Xba* I	*Xho* I
Xma I			

Vector Maps

pGEMEX™-1

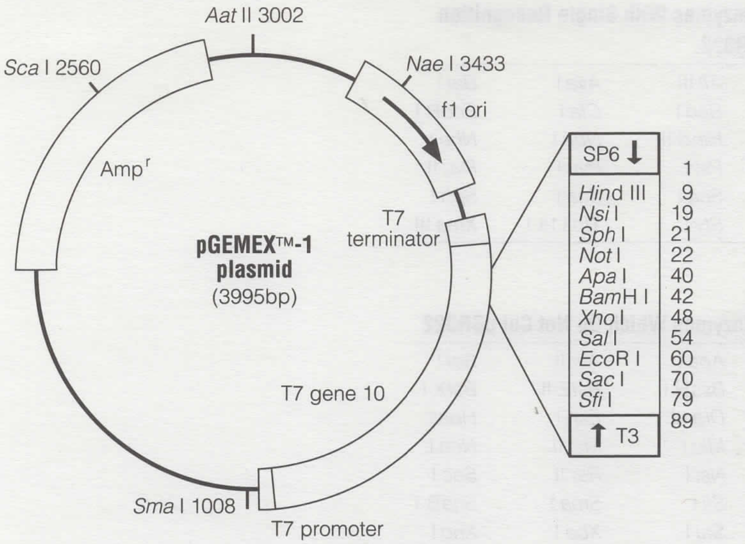


Figure 48. pGEMEX-1 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T3 RNA polymerase transcription initiation site 89
   c. SP6 RNA polymerase promoter 3979-3995
   d. T3 RNA polymerase promoter 90-106
   e. multiple cloning sites 9-84
   f. T7 RNA polymerase transcription initiation site 968
   g. T7 RNA polymerase promoter 969-985
   h. T7 gene 10 start codon 905
   i. T7 terminator region 3841-3931
   j. phage f1 region 3306-3761
   k. β-lactamase (Ampʳ) coding region 2007-2867
2. Specialized applications:
   a. high-level, in-frame expression of inserts subcloned from the λgt11 vector
   b. ssDNA production
   c. transcription *in vitro* from dual opposed promoters
3. Use the T3 primer to sequence ssDNA produced by the pGEMEX-1 vector.

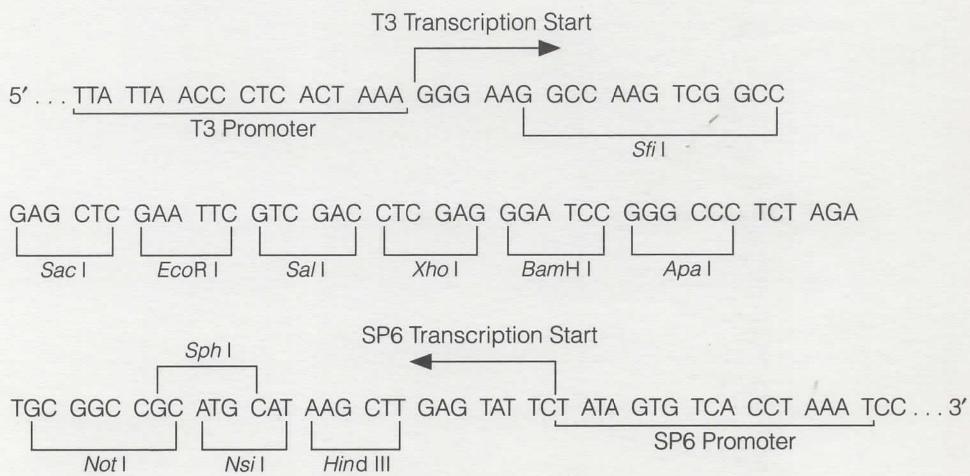


Figure 49. In-frame sequence of the pGEMEX-1 vector multiple cloning region. The sequence shown corresponds to the in-frame sequence of gene 10 fusion protein mRNA synthesized by T7 RNA polymerase. This sequence also corresponds to RNA synthesized by T3 RNA polymerase, is complementary to RNA synthesized by SP6 RNA polymerase, and is complementary to single-stranded DNA generated from the f1 origin.

Technical
Services:
800-356-9526

Table 28. pGEMEX-1 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	3002
Acc I	2	55, 539
Acc III	1	3836
Acy I	2	2617, 2999
Aha II	2	2617, 2999
Alu I	18	11, 68, 267, 1129, 1355, 1445, 1491, 1748, 2269, 2369, 2432, 3111, 3130, 3449, 3488, 3706, 3939, 3973
Alw44 I	3	1501, 2747, 3244
Apa I	1	40
Asu I	10	36, 37, 2122, 2201, 2218, 2440, 3056, 3542, 3809, 3887
Asu II	0	
Ava I	2	48, 1006
Ava II	2	2218, 2440
Bal I	1	113
BamH I	1	42
Ban I	3	126, 2028, 3495
Ban II	3	40, 70, 3465
Bbu I	1	21
Bbv I	22	216, 229, 234, 310, 517, 678, 856, 998, 1079, 1097, 1516, 1606, 1609, 1815, 2118, 2509, 3120, 3366, 3764, 3936, 3939, 3972
Bcl I	0	
Bgl I	3	79, 2200, 3774
Bgl II	1	1000
Bsm I	0	
BspH I	3	1907, 2915, 3020
BspM I	0	
BspM II	1	3836
Bsp1286 I	7	40, 70, 1505, 2666, 2751, 3248, 3465
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	22	370, 1036, 1064, 1097, 1367, 1434, 1534, 1708, 1817, 2210, 2303, 2590, 2972, 3072, 3175, 3310, 3323, 3332, 3354, 3380, 3388, 3782
Cfr I	5	22, 71, 111, 1026, 2468
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	17	201, 336, 399, 428, 483, 531, 543, 696, 720, 1462, 1871, 2037, 2577, 3003, 3238, 3914, 3935
Dpn I	19	44, 122, 996, 1002, 1755, 1830, 1841, 1849, 1927, 1939, 2044, 2385, 2403, 2449, 2707, 2724, 2760, 3801, 3968
Dra I	3	1946, 1965, 2657
Dra II	3	36, 3056, 3887
Dra III	1	3539
Eco47 III	0	
Eco52 I	2	22, 71
EcoR I	1	60
EcoR V	0	
Fnu4H I	35	22, 25, 218, 230, 248, 299, 326, 347, 389, 506, 692, 845, 1012, 1093, 1111, 1114, 1232, 1387, 1530, 1595, 1598, 1804, 2132, 2471, 2498, 2822, 3109, 3218, 3319, 3333, 3355, 3778, 3925, 3928, 3961
Fok I	5	486, 2046, 2227, 2514, 3157
Fsp I	2	2302, 3781
Hae II	4	1065, 1435, 3381, 3389
Hae III	19	24, 38, 73, 82, 113, 391, 1028, 1202, 1213, 1231, 1665, 2123, 2203, 2470, 3057, 3544, 3686, 3811, 3888
Hga I	6	560, 1298, 1876, 2606, 3164, 3314
HgiA I	5	70, 1505, 2666, 2751, 3248
Hha I	22	370, 1036, 1064, 1097, 1367, 1434, 1534, 1708, 1817, 2210, 2303, 2590, 2972, 3072, 3175, 3310, 3323, 3332, 3354, 3380, 3388, 3782
Hinc II	1	56
Hind II	1	56
Hind III	1	9
Hinf I	10	254, 520, 975, 1022, 1087, 1162, 1558, 2075, 3584, 3606
Hpa I	0	
Hpa II	21	40, 124, 302, 475, 677, 1007, 1394, 1541, 1567, 1757, 2161, 2195, 2262, 2372, 2592, 2614, 3115, 3149, 3432, 3837, 3964
Hph I	12	156, 515, 633, 794, 1923, 2150, 2566, 2772, 2807, 3091, 3100, 3536
Kpn I	1	130
Mae I	8	31, 898, 943, 1682, 1935, 2270, 3383, 3904
Mae II	15	181, 463, 553, 595, 654, 747, 1890, 2306, 2679, 2999, 3425, 3535, 3578, 3590, 3749
Mae III	15	275, 464, 768, 1543, 1606, 1722, 2005, 2336, 2394, 2547, 2735, 3123, 3346, 3358, 3984
Mbo I	19	42, 120, 994, 1000, 1753, 1828, 1839, 1847, 1925, 1937, 2042, 2383, 2401, 2447, 2705, 2722, 2758, 3799, 3966
Mbo II	8	1058, 1849, 1920, 2675, 2753, 2862, 3397, 3806
Mlu I	0	
Mnl I	28	26, 43, 57, 88, 139, 177, 444, 527, 585, 658, 767, 938, 1035, 1086, 1294, 1369, 1618, 2018, 2099, 2247, 2453, 3046, 3106, 3509, 3822, 3859, 3877, 3879
Msp I	21	40, 124, 302, 475, 677, 1007, 1394, 1541, 1567, 1757, 2161, 2195, 2262, 2372, 2592, 2614, 3115, 3149, 3432, 3837, 3964
Nae I	1	3433
Nar I	0	
Nci I	10	40, 677, 1007, 1008, 1567, 2263, 2592, 2614, 3115, 3150
Nco I	0	
Nde I	2	904, 3251
Nhe I	1	897
Nla III	12	21, 756, 897, 1191, 1911, 2402, 2412, 2490, 2526, 2919, 3024, 3108
Nla IV	15	38, 44, 128, 681, 1219, 1258, 2030, 2124, 2165, 2376, 2966, 3464, 3476, 3497, 3889
Not I	1	22
Nsi I	1	19
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	2	2450, 3802
Pvu II	0	
Rsa I	7	128, 425, 750, 779, 872, 2560, 3236
Sac I	1	70
Sac II	0	
Sal I	1	54
Sau3A I	19	42, 120, 994, 1000, 1753, 1828, 1839, 1847, 1925, 1937, 2042, 2383, 2401, 2447, 2705, 2722, 2758, 3799, 3966
Sau96 I	10	36, 37, 2122, 2201, 2218, 2440, 3056, 3542, 3809, 3887
Sca I	1	2560
ScrF I	14	40, 677, 1007, 1008, 1215, 1336, 1349, 1567, 2263, 2592, 2614, 3115, 3150, 3829
SfaN I	10	36, 248, 1284, 2336, 2527, 2776, 3135, 3229, 3265, 3305
Sfi I	1	79
Sin I	2	2218, 2440
Sma I	1	1008
Spe I	0	
Sph I	1	21
Spo I	0	
Ssp I	3	2884, 3720, 3744
Stu I	0	
Sty I	2	862, 3891
Taq I	11	49, 55, 64, 119, 436, 664, 997, 1250, 1287, 2731, 3501
Tha I	16	571, 991, 1034, 1036, 1234, 1815, 2145, 2588, 2970, 3070, 3072, 3175, 3308, 3332, 3352, 3728
Tth111 I	0	
Tth111 II	4	630, 1777, 1784, 1816
Xba I	2	30, 942
Xho I	1	48
Xho II	8	42, 1000, 1828, 1839, 1925, 1937, 2705, 2722
Xma I	1	1008
Xma III	2	22, 71

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pGEMEX™-2

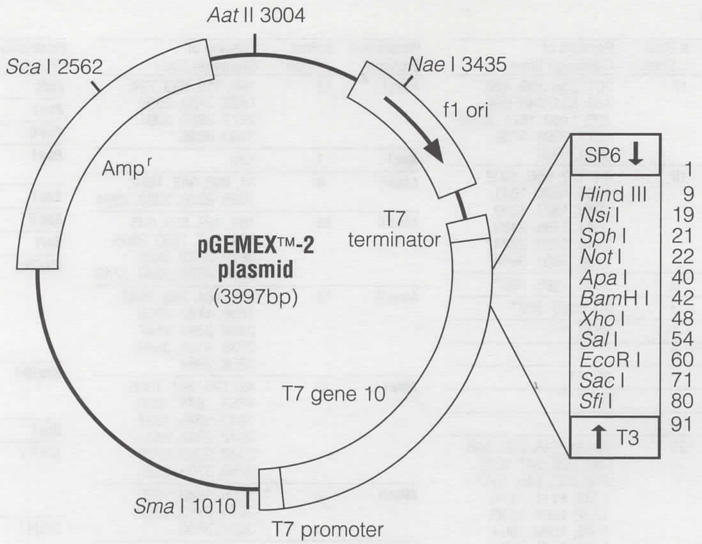


Figure 50. pGEMEX-2 vector circle map.

Notes:

1. Sequence reference points:
   a. SP6 RNA polymerase transcription initiation site 1
   b. T3 RNA polymerase transcription initiation site 91
   c. SP6 RNA polymerase promoter 3981-3997
   d. T3 RNA polymerase promoter 92-109
   e. multiple cloning sites 9-85
   f. T7 RNA polymerase transcription initiation site 970
   g. T7 RNA polymerase promoter 971-987
   h. T7 gene 10 start codon 907
   i. T7 terminator region 3843-3933
   j. Phage f1 region 3308-3763
   k. β-lactamase (Amp^r) coding region 2009-2869
2. Specialized applications:
   a. high-level, in-frame expression of inserts subcloned from the λgt11 *Sfi-Not* vector
   b. ssDNA production
   c. transcription *in vitro* from dual opposed promoters
3. Use the T3 primer to sequence ssDNA produced by the pGEMEX-2 vector.

T3 Transcription Start

5′ . . . TTA TTA ACC CTC ACT AAA GGG AAG

T3 Promoter

GGC CAA GTC GGC CGA GCT CGG AAT TCG TCG ACC TCG AGG GAT CCG GGC CCT

Sfi I *Sac* I *EcoR* I *Sal* I *Xho* I *BamH* I *Apa* I

Sph I SP6 Transcription Start

CTA GAC GCG GCC GCA TGC ATA AGC TTG AGT ATT CTA TAG TGT CAC CTA AAT CC . . . 3′

Not I *Nsi* I *Hind* III SP6 Promoter

Figure 51. In-frame sequence of the pGEMEX-2 vector multiple cloning region. The sequence shown corresponds to the in-frame sequence of gene 10 fusion protein mRNA synthesized by T7 RNA polymerase. This sequence also corresponds to RNA synthesized by T3 RNA polymerase, is complementary to RNA synthesized by SP6 RNA polymerase, and is complementary to single-stranded DNA generated from the f1 origin.

Table 29. pGEMEX-2 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat I	1	3004
Acc I	2	55, 541
Acc III	1	3838
Acy I	2	2619, 3001
Aha II	2	2619, 3001
Alu I	18	11, 69, 269, 1131, 1357, 1447, 1493, 1750, 2271, 2371, 2434, 3113, 3132, 3451, 3490, 3708, 3941, 3975
Alw44 I	3	1503, 2749, 3249
Apa I	1	40
Asu I	11	36, 37, 82, 2124, 2203, 2220, 2442, 3058, 3544, 3811, 3889
Asu II	0	See **Csp45 I**
Ava I	2	48, 1008
Ava II	2	2220, 2442
Bal I	1	115
BamH I	1	42
Ban I	3	128, 2030, 3497
Ban II	3	40, 71, 3467
Bbu I	1	21
Bbv I	22	218, 231, 236, 312, 519, 680, 858, 1000, 1081, 1099, 1518, 1608, 1611, 1817, 2120, 2511, 3122, 3368, 3766, 3938, 3941, 3974
Bcl I	0	
Bgl I	3	80, 2202, 3776
Bgl II	1	1002
Bsm I	0	
Bsp1286 I	7	40, 71, 1507, 2668, 2753, 3250, 3467
BspH I	3	1909, 2917, 3022
BspM I	0	
BspM II	1	3838
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	22	372, 1038, 1066, 1099, 1369, 1436, 1536, 1710, 1819, 2212, 2305, 2592, 2974, 3074, 3177, 3312, 3325, 3334, 3356, 3382, 3390, 3784
Cfr I	5	22, 72, 113, 1028, 2470
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	17	203, 338, 401, 430, 485, 533, 545, 698, 722, 1464, 1873, 2039, 2579, 3005, 3240, 3916, 3937
Dpn I	19	44, 124, 998, 1004, 1757, 1832, 1843, 1851, 1929, 1941, 2046, 2387, 2405, 2451, 2709, 2726, 2762, 3803, 3970
Dra I	3	1948, 1967, 2659
Dra II	3	36, 3058, 3889
Dra III	1	3541
Eco47 III	0	
Eco52 I	2	22, 72
EcoR I	1	60
EcoR V	0	
Fnu4H I	35	22, 25, 220, 232, 250, 301, 328, 349, 391, 508, 694, 847, 1014, 1095, 1113, 1116, 1234, 1389, 1532, 1597, 1600, 1806, 2134, 2473, 2500, 2824, 3111, 3220, 3321, 3335, 3357, 3780, 3927, 3930, 3963
Fok I	5	488, 2048, 2229, 2516, 3159
Fsp I	2	2304, 3783
Hae II	4	1067, 1437, 3383, 3391
Hae III	19	24, 38, 74, 83, 115, 393, 1030, 1204, 1215, 1233, 1667, 2125, 2205, 2472, 3059, 3546, 3688, 3813, 3890
Hga I	7	16, 562, 1300, 1878, 2608, 3166, 3316
HgiA I	5	71, 1507, 2668, 2753, 3250
Hha I	22	372, 1038, 1066, 1099, 1369, 1436, 1536, 1710, 1819, 2212, 2305, 2592, 2974, 3074, 3177, 3312, 3325, 3334, 3356, 3382, 3390, 3784
Hinc II	1	56
Hind II	1	56
Hind III	1	9
Hinf I	10	256, 522, 977, 1024, 1089, 1164, 1560, 2077, 3586, 3608
Hpa I	0	
Hpa II	21	40, 126, 304, 477, 679, 1009, 1396, 1543, 1569, 1759, 2163, 2197, 2264, 2374, 2594, 2616, 3117, 3151, 3434, 3839, 3966
Hph I	12	158, 517, 635, 796, 1925, 2152, 2568, 2774, 2809, 3093, 3102, 3538
Kpn I	1	132
Mae I	8	31, 900, 945, 1684, 1937, 2272, 3385, 3906
Mae II	15	183, 465, 555, 597, 656, 749, 1892, 2308, 2681, 3001, 3427, 3537, 3580, 3592, 3751
Mae III	15	277, 466, 770, 1545, 1608, 1724, 2007, 2338, 2396, 2549, 2737, 3125, 3348, 3360, 3986
Mbo I	19	42, 122, 996, 1002, 1755, 1830, 1841, 1849, 1927, 1939, 2044, 2385, 2403, 2449, 2707, 2724, 2760, 3801, 3968
Mbo II	8	1060, 1851, 1922, 2677, 2755, 2864, 3399, 3808
Mlu I	0	
Mnl I	28	26, 43, 57, 90, 141, 179, 446, 529, 587, 660, 769, 940, 1037, 1088, 1296, 1371, 1620, 2020, 2101, 2249, 2455, 3048, 3108, 3511, 3824, 3861, 3879, 3881
Msp I	21	40, 126, 304, 477, 679, 1009, 1396, 1543, 1569, 1759, 2163, 2197, 2264, 2374, 2594, 2616, 3117, 3151, 3434, 3839, 3966
Nae I	1	3435
Nar I	0	
Nci I	10	40, 679, 1009, 1010, 1569, 2265, 2594, 2616, 3117, 3152
Nco I	0	
Nde I	2	906, 3253
Nhe I	1	899
Nla III	12	21, 758, 899, 1193, 1913, 2404, 2414, 2492, 2528, 2921, 3026, 3110
Nla IV	15	38, 44, 130, 683, 1221, 1260, 2032, 2126, 2167, 2378, 2968, 3466, 3478, 3499, 3891
Not I	1	22
Nsi I	1	19
PflM I	0	
PpuM I	0	
Pst I	0	
Pvu I	2	2452, 3804
Pvu II	0	
Rsa I	7	130, 427, 752, 781, 874, 2562, 3238
Sac I	1	71
Sac II	0	
Sal I	1	54
Sau3A I	19	42, 122, 996, 1002, 1755, 1830, 1841, 1849, 1927, 1939, 2044, 2385, 2403, 2449, 2707, 2724, 2760, 3801, 3968
Sau96 I	11	36, 37, 82, 2124, 2203, 2220, 2442, 3058, 3544, 3811, 3889
Sca I	1	2562
ScrF I	14	40, 679, 1009, 1010, 1217, 1338, 1351, 1569, 2265, 2594, 2616, 3117, 3152, 3831
SfaN I	9	250, 1286, 2338, 2529, 2778, 3137, 3231, 3267, 3307
Sfi I	1	80
Sin I	2	2220, 2442
Sma I	1	1010
Spe I	0	
Sph I	1	21
Spo I	0	
Ssp I	3	2886, 3722, 3746
Stu I	0	
Sty I	2	864, 3893
Taq I	10	49, 55, 121, 438, 666, 999, 1252, 1289, 2733, 3503
Tha I	17	27, 573, 993, 1036, 1038, 1236, 1817, 2147, 2590, 2972, 3072, 3074, 3177, 3310, 3334, 3354, 3730
Tth111 I	0	
Tth111 II	4	632, 1779, 1786, 1818
Xba I	2	30, 944
Xho I	1	48
Xho II	8	42, 1002, 1830, 1841, 1927, 1939, 2707, 2724
Xma I	1	1010
Xma III	2	22, 72

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSELECT™-1

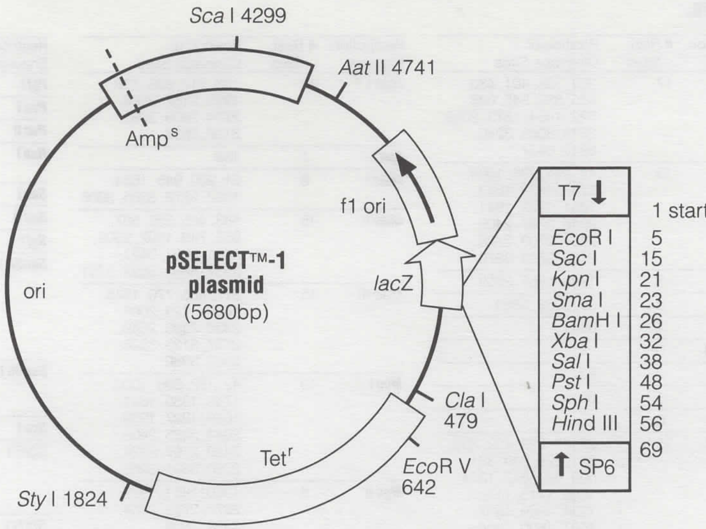


Figure 52. pSELECT-1 vector circle map.

Notes:

1. Sequence reference points
   a. T7 RNA polymerase transcription initiation site 1
   b. SP6 RNA polymerase transcription initiation site 69
   c. T7 RNA polymerase promoter 5654-5680
   d. SP6 RNA polymerase promoter 70-86
   e. multiple cloning sites 5-61
   f. *lacZ* start codon 108
   g. *lac* operon sequences 5501-5661; 94-323
   h. *lac* operator 128-144
   i. β-lactamase (Amp^r) coding region 3750-4606
   j. phage f1 region 5045-5500
   k. binding site of pUC/M13 forward sequencing primer 5621-5637
   l. binding site of pUC/M13 reverse sequencing primer 104-120
2. Specialized applications:
   a. site-directed *in vitro* mutagenesis
   b. ssDNA production
   c. blue/white screening for recombinants
   d. transcription *in vitro* from dual opposed promoters
3. Use the T7 or pUC/M13 forward primers to sequence ssDNA produced by the pSELECT-1 vector.

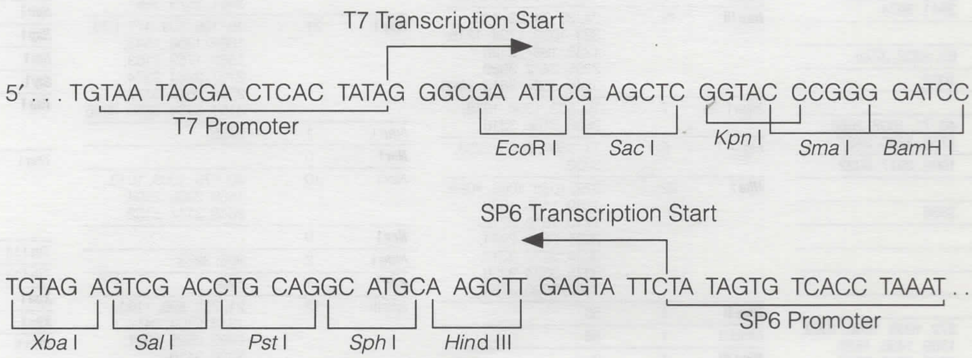


Figure 53. pSELECT-1 plasmid promoter and multiple cloning site sequence. The sequence shown is complementary to the ssDNA produced upon infection with helper phage. This sequence corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

Table 30. pSELECT-1 Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	4741
Acc I	2	39, 2702
Acc III	1	2119
Acy I	6	869, 890, 1004, 1661, 4356, 4738
Aha II	6	869, 890, 1004, 1661, 4356, 4738
Alu I	30	13, 58, 88, 110, 205, 269, 387, 471, 486, 1142, 1545, 2455, 2512, 2523, 2572, 2591, 2872, 3098, 3188, 3234, 3491, 4012, 4108, 4171, 4850, 4869, 5099, 5317, 5356, 5570
Alw44 I	4	2746, 3244, 4486, 4983
Apa I	0	
Asu I	17	627, 979, 1254, 1342, 1591, 1715, 1894, 1936, 2215, 2404, 3865, 3944, 3961, 4179, 4795, 5260, 5548
Asu II	0	See **Csp45 I**
Ava I	2	21, 1880
Ava II	8	1254, 1342, 1591, 1894, 1936, 2215, 3961, 4179
Bal I	1	1901
BamH I	1	26
Ban I	12	17, 189, 531, 574, 868, 889, 1003, 1221, 1660, 1744, 3771, 5306
Ban II	4	15, 930, 944, 5344
Bbu I	1	54
Bbv I	27	256, 337, 355, 668, 1057, 1240, 1873, 1897, 2001, 2004, 2127, 2510, 2532, 2581, 2678, 2822, 2840, 3259, 3349, 3352, 3558, 3861, 4248, 4859, 5435, 5503, 5576
Bcl I	0	
Bgl I	5	1390, 1624, 3943, 4063, 5513
Bgl II	0	
Bsm I	1	1814
Bsp1286 I	13	15, 735, 930, 944, 1046, 1633, 1924, 2750, 3248, 4405, 4490, 4987, 5344
*Bsp*H I	4	944, 3650, 4654, 4759
*Bsp*M I	2	51, 1509
*Bsp*M II	1	2119
BssH II	0	
BstE II	0	
BstX I	0	
Cfo I	45	229, 294, 322, 355, 558, 690, 718, 871, 892, 952, 1006, 1106, 1158, 1233, 1273, 1404, 1663, 1814, 1876, 1912, 2102, 2185, 2533, 2636, 2666, 2807, 2840, 3110, 3177, 3277, 3451, 3560, 3953, 4046, 4379, 4711, 4811, 4914, 5419, 5427, 5453, 5475, 5484, 5497, 5521
Cfr I	8	284, 750, 854, 986, 1394, 1899, 4207, 5650
Cla I	1	479
Csp I	0	
Csp45 I	0	
Dde I	9	2036, 2198, 2740, 3205, 3614, 3780, 4316, 4742, 4977
Dpn I	23	28, 805, 923, 1282, 1554, 1585, 1600, 1917, 2124, 3498, 3573, 3584, 3592, 3670, 3682, 3787, 4124, 4142, 4188, 4446, 4463, 4499, 5540
Dra I	3	3689, 3708, 4396
Dra II	4	979, 1894, 1936, 4795
Dra III	1	5269
Eco47 III	4	689, 951, 1232, 2184
Eco52 I	1	1394
EcoR I	1	5
EcoR V	1	642
*Fnu*4H	53	270, 351, 369, 372, 682, 753, 756, 1034, 1037, 1071, 1178, 1229, 1394, 1479, 1562, 1619, 1664, 1743, 1862, 1865, 1872, 1886, 2015, 2018, 2141, 2222, 2521, 2524, 2570, 2667, 2720, 2836, 2854, 2857, 2975, 3130, 3273, 3338, 3341, 3547, 3875, 4064, 4210, 4237, 4332, 4561, 4848, 4957, 5449, 5471, 5485, 5517, 5590
Fok I	14	553, 601, 1455, 1500, 2149, 2211, 2289, 2477, 2618, 3789, 3970, 4253, 4896, 5596
Fsp I	5	717, 1813, 1911, 4045, 5520
Hae II	15	323, 691, 872, 893, 953, 1007, 1107, 1234, 1664, 2103, 2186, 2808, 3178, 5420, 5428
Hae III	27	286, 629, 752, 856, 980, 988, 1052, 1286, 1375, 1396, 1447, 1504, 1717, 1901, 2405, 2945, 2956, 2974, 3408, 3866, 3946, 4209, 4796, 5119, 5261, 5550, 5652
Hga I	12	854, 1408, 1440, 1684, 1834, 2468, 2625, 3041, 3619, 4345, 4903, 5486
*Hgi*A I	10	15, 735, 1046, 1633, 1924, 2750, 3248, 4405, 4490, 4987
Hha I	45	229, 294, 322, 355, 558, 690, 718, 871, 892, 952, 1006, 1106, 1158, 1233, 1273, 1404, 1663, 1814, 1876, 1912, 2102, 2185, 2533, 2636, 2666, 2807, 2840, 3110, 3177, 3277, 3451, 3560, 3953, 4046, 4379, 4711, 4811, 4914, 5419, 5427, 5453, 5475, 5484, 5497, 5521
Hinc II	2	40, 4360
Hind II	2	40, 4360
Hind III	1	56
Hinf I	18	36, 280, 345, 420, 831, 1087, 1307, 1461, 1759, 1980, 2486, 2830, 2905, 3301, 3818, 5196, 5218, 5670
Hpa I	0	
Hpa II	31	22, 163, 616, 625, 842, 857, 866, 989, 1149,
Hpa II *(cont.)*		1225, 1385, 1475, 1713, 1739, 1940, 2120, 2267, 2576, 2610, 3137, 3284, 3310, 3500, 3904, 3938, 4005, 4111, 4353, 4854, 4888, 5371
Hph I	16	69, 573, 855, 900, 1754, 1975, 2552, 2561, 3666, 3893, 4305, 4511, 4546, 4830, 4839, 5270
Kpn I	1	21
Mae I	7	33, 685, 1944, 3425, 3678, 4013, 5420
Mae II	16	1356, 1412, 2001, 2025, 2255, 2683, 3633, 4049, 4418, 4738, 5054, 5213, 5225, 5268, 5378, 5636
Mae III	23	75, 579, 667, 1335, 1602, 2262, 2285, 2371, 2584, 2679, 3286, 3349, 3465, 3748, 4075, 4133, 4286, 4474, 4862, 5442, 5454, 5609, 5629
Mbo I	23	26, 803, 921, 1280, 1552, 1583, 1598, 1915, 2122, 3496, 3571, 3582, 3590, 3668, 3680, 3785, 4122, 4140, 4186, 4444, 4461, 4497, 5538
Mbo II	13	316, 931, 1185, 1456, 2048, 2801, 3592, 3663, 4414, 4492, 4601, 5409, 5545
Mlu I	0	
Mnl I	31	40, 293, 344, 580, 640, 1063, 1244, 1330, 1428, 1632, 1693, 1731, 1758, 1926, 2258, 2316, 2354, 2537, 2567, 2829, 3037, 3112, 3361, 3761, 3842, 3990, 4192, 4785, 4845, 5296, 5561
Msp I	31	22, 163, 616, 625, 842, 857, 866, 989, 1149, 1225, 1385, 1475, 1713, 1739, 1940, 2120, 2267, 2576, 2610, 3137, 3284, 3310, 3500, 3904, 3938, 4005, 4111, 4353, 4854, 4888, 5371
Nae I	5	858, 1226, 1386, 1740, 5372
Nar I	4	869, 890, 1004, 1661
Nci I	14	22, 23, 626, 990, 1714, 1940, 2268, 2576, 2611, 3310, 4006, 4353, 4854, 4889
Nco I	0	
Nde I	2	2753, 4990
Nhe I	1	684
Nla III	30	54, 103, 449, 463, 810, 948, 1021, 1167, 1206, 1392, 1509, 1637, 1652, 1709, 1916, 2050, 2275, 2339, 2404, 2569, 2674, 2934, 3654, 4141, 4151, 4229, 4265, 4658, 4763, 4847
Nla IV	29	19, 28, 191, 533, 576, 786, 870, 891, 981, 1005, 1223, 1344, 1662, 1711, 1746, 1781, 1895, 1938, 2217, 2962, 3001, 3773, 3867, 3908, 4115, 4705, 5308, 5329, 5341
Not I	0	
Nsi I	0	
*Pfl*M I	2	1776, 1825
*Ppu*M I	2	1894, 1936
Pst I	1	48
Pvu I	2	4189, 5541
Pvu II	3	269, 2523, 5570
Rsa I	5	19, 620, 2738, 4299, 4975
Sac I	1	15
Sac II	0	
Sal I	1	38
Sau3A I	23	26, 803, 921, 1280, 1552, 1583, 1598, 1915, 2122, 3496, 3571, 3582, 3590, 3668, 3680, 3785, 4122, 4140, 4186, 4444, 4461, 4497, 5538
Sau96 I	17	627, 979, 1254, 1342, 1591, 1715, 1894, 1936, 2215, 2404, 3865, 3944, 3961, 4179, 4795, 5260, 5548
Sca I	1	4299
*Scr*F I	22	22, 23, 185, 586, 626, 990, 1515, 1714, 1898, 1940, 2268, 2576, 2611, 2958, 3079, 3092, 3310, 4006, 4353, 4854, 4889, 5617
*Sfa*N I	26	579, 668, 692, 857, 869, 1103, 1478, 1490, 1885, 2127, 2137, 2233, 2311, 2374, 2596, 2731, 2767, 2807, 3027, 4075, 4266, 4515, 4874, 4968, 5004, 5044
Sfi I	0	
Sin I	8	1254, 1342, 1591, 1894, 1936, 2215, 3961, 4179
Sma I	1	23
Spe I	0	
Sph I	1	54
Spo I	1	1429
Ssp I	3	4623, 5061, 5085
Stu I	0	
Sty I	1	1824
Taq I	9	9, 39, 479, 794, 1582, 1723, 3030, 4470, 5302
Tha I	32	292, 294, 802, 1158, 1273, 1402, 1429, 1434, 1495, 1561, 1690, 1700, 1845, 1871, 1993, 2090, 2462, 2531, 2533, 2636, 2977, 3558, 3888, 4377, 4709, 4809, 4811, 4914, 5077, 5453, 5473, 5497
*Tth*111 I	1	2677
Xba I	1	32
Xho I	0	
Xho II	8	26, 2122, 3571, 3582, 3668, 3680, 4444, 4461
Xma I	1	21
Xma III	1	1394

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pCAT®-Basic

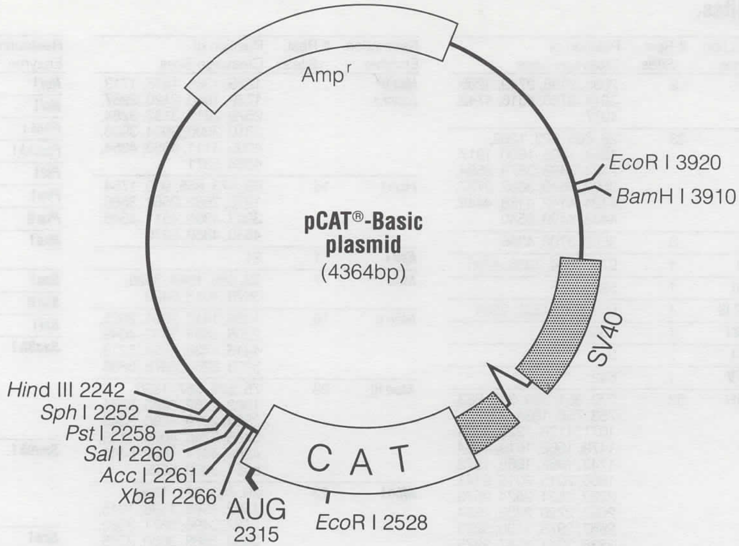


Figure 54. pCAT-Basic vector circle map.

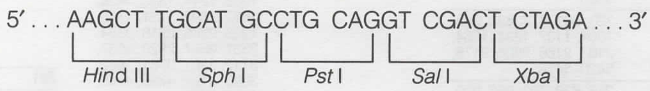


Figure 55. pCAT-Basic plasmid multiple cloning sequence upstream from the CAT gene.

Notes:

1. Sequence reference points:
   a. multiple cloning sites (*Hind* III - *Xba* I) 2242-2271
   b. SV40 large T antigen region 2975-3677
   c. CAT gene start site 2315
   d. CAT gene stop site 2974
   e. β-lactamase (Amp^r) coding region 209-1069
2. Specialized application:
   a. analysis of eukaryotic promoter and enhancer sequences

Promega

Table 31. pCAT-Basic Vector Restrictions Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	77
Acc I	1	2261
Acc III	1	2524
Acy I	3	74, 456, 4082
Aha II	3	74, 456, 4082
Alu I	22	643, 706, 806, 1327, 1584, 1720, 1946, 2063, 2222, 2244, 2280, 2300, 2309, 2428, 2557, 3132, 3461, 3615, 3795, 4012, 4296, 4315
Alw44 I	3	324, 1570, 4139
Apa I	0	
Asu I	6	16, 632, 854, 871, 950, 4031
Asu II	0	See **Csp45 I**
Ava I	0	
Ava II	2	632, 854
Bal I	1	2795
BamH I	1	3910
Ban I	4	1043, 2139, 2994, 4081
Ban II	0	
Ban III	0	
Bbu I	1	2252
Bbv I	14	562, 953, 1256, 1462, 1465, 1555, 1974, 1992, 2072, 3448, 3804, 4002, 4205, 4302
Bcl I	0	
Bgl I	2	878, 4072
Bgl II	0	
Bsm I	5	2521, 2928, 3250, 3767, 3860
Bsp1286 I	5	328, 413, 1574, 2999, 4143
BspH I	3	51, 156, 1164
BspM I	1	2247
BspM II	1	2524
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	17	105, 437, 774, 867, 1260, 1369, 1543, 1643, 1710, 1980, 2013, 2040, 2105, 4084, 4223, 4253, 4356
Cfr I	4	603, 2044, 2793, 3928
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	12	69, 495, 1035, 1201, 1610, 2276, 2301, 2749, 3135, 3274, 3386, 4146
Dpn I	18	315, 351, 368, 626, 672, 690, 1031, 1136, 1148, 1226, 1234, 1245, 1320, 2274, 3065, 3675, 3912, 4042
Dra I	7	418, 1110, 1129, 2448, 2787, 3128, 3718
Dra II	1	16
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	2	2528, 3920
EcoR V	0	
Fnu4H I	22	252, 481, 576, 603, 942, 1270, 1476, 1479, 1544, 1687, 1842, 1960, 1963,1981, 2061, 2870, 3462, 3793, 3991, 4168, 4219, 4316
Fok I	7	557, 844, 1025, 2508, 3054, 3982, 4265
Fsp I	1	773
Hae II	3	1644, 2014, 4085
Hae III	15	18, 605, 872, 952, 1410, 1844, 1862, 1873, 2046, 2441, 2486, 2708, 2795, 3930, 4032
Hga I	4	464, 1194, 1772, 4257
HgiA I	4	328, 413, 1574, 4143
Hha I	17	105, 437, 774, 867, 1260, 1369, 1543, 1643, 1710, 1980, 2013, 2040, 2105, 4084, 4223, 4253, 4356
Hinc II	2	2262, 3779
Hind II	2	2262, 3779
Hind III	1	2242
Hinf I	11	997, 1514, 1910, 1985, 2049, 2263, 2878, 3181, 3205, 3314, 3397
Hpa I	1	3779
Hpa II	17	459, 701, 811, 878, 912, 1316, 1506, 1532, 1679, 2167, 2483, 2525, 2653, 3061, 3917, 4275, 4309
Hph I	14	269, 304, 510, 926, 1153, 2564, 2570, 2572, 2708, 2764, 2776, 2818, 4327, 4336
Kpn I	0	
Mae I	7	803, 1138, 1391, 2267, 3291, 3669, 3865
Mae II	8	74, 394, 767, 1183, 2615, 2790, 3944, 4202
Mae III	18	335, 523, 676, 734, 1065, 1348, 1464, 1527, 2122, 2585, 2690, 3094, 3499, 3804, 3948, 3968, 4203, 4298
Mbo I	18	313, 349, 366, 624, 670, 688, 1029, 1134, 1146, 1224, 1232, 1243, 1318, 2272, 3063, 3673, 3910, 4040
Mbo II	12	18, 214, 323, 401, 1156, 1227, 2018, 2803, 3290, 3355, 3368, 4038
Mlu I	0	
Mnl I	22	29, 622, 828, 976, 1057, 1457, 1706, 1781, 1989, 2039, 2137, 2262, 2373, 3249, 3294, 3342, 3694, 3735, 3744, 4021, 4320, 4350
Msp I	17	459, 701, 811, 878, 912, 1316, 1506, 1532, 1679, 2167, 2483, 2525, 2653, 3061, 3917, 4275, 4309
Nae I	0	
Nar I	1	4082
Nci I	6	460, 811, 1507, 3917, 4275, 4310
Nco I	1	2829
Nde I	1	4134
Nhe I	0	
Nla III	18	55, 160, 553, 589, 667, 677, 1168, 1888, 2233, 2252, 2603, 2833, 2894, 2915, 3404, 3906, 4217, 4322
Nla IV	12	109, 699, 910, 951, 1045, 1817, 1856, 2141, 2996, 3078, 3912, 4083
Not I	0	
Nsi I	0	
PflM I	2	2760, 3215
PpuM I	0	
Pst I	1	2258
Pvu I	2	627, 4043
Pvu II	3	2063, 2428, 4012
Rsa I	6	515, 2407, 2945, 3607, 4151, 4185
Sac I	0	
Sac II	0	
Sal I	1	2260
Sau3A I	18	313, 349, 366, 624, 670, 688, 1029, 1134, 1146, 1224, 1232, 1243, 1318, 2272, 3063, 3673, 3910, 4040
Sau96 I	6	16, 632, 854, 871, 950, 4031
Sca I	2	515, 2945
ScrF I	14	460, 811, 1507, 1725, 1738, 1859, 2146, 2705, 2761, 3009, 3917, 3964, 4275, 4310
SfaN I	11	295, 544, 735, 1787, 2371, 2856, 3832, 4078, 4118, 4154, 4287
Sfi I	0	
Sin I	2	632, 854
Sma I	0	
Spe I	0	
Sph I	1	2252
Spo I	1	2918
Ssp I	4	191, 2840, 3494, 3647
Sst I	0	
Sst II	0	
Stu I	0	
Sty I	2	2829, 3363
Taq I	3	342, 1786, 2261
Tha I	10	105, 437, 930, 1260, 1841, 2038, 2040, 4251, 4354, 4356
Tth111 I	1	4209
Xba I	1	2266
Xho I	0	
Xho II	9	349, 366, 1134, 1146, 1232, 1243, 2272, 3063, 3910
Xma I	0	
Xma III	0	
Xmn I	1	396

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pCAT®-Enhancer

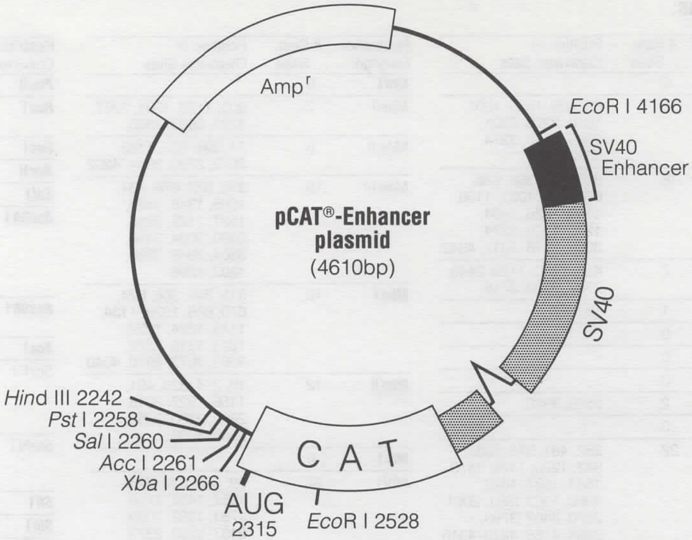


Figure 56. pCAT-Enhancer vector circle map.

Notes:

1. Sequence reference sites:
   a. multiple cloning sites (*Hind* III - *Xba* I) 2242-2271
   b. SV40 enhancer region 3916-4152
   c. CAT gene start site 2315
   d. CAT gene stop site 2974
   e. β-lactamase (Amp^r) coding region 209-1069
2. Specialized application:
   a. analysis of eukaryotic promoter sequences

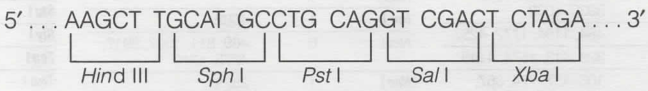


Figure 57. pCAT-Enhancer plasmid multiple cloning sequence upstream from the CAT gene.

Table 32. pCAT-Enhancer Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	77
Acc I	1	2261
Acc III	1	2524
Acy I	3	74, 456, 4328
Aha II	3	74, 456, 4328
Alu I	22	643, 706, 806, 1327, 1584, 1720, 1946, 2063, 2222, 2244, 2280, 2300, 2309, 2428, 2557, 3132, 3461, 3615, 3795, 4258, 4542, 4561
Alw44 I	3	324, 1570, 4385
Apa I	0	
Asu I	6	16, 632, 854, 871, 950, 4277
Asu II	0	See **Csp45 I**
Ava I	0	
Ava II	2	632, 854
Bal I	1	2795
BamH I	0	
Ban I	4	1043, 2139, 2994, 4327
Ban II	0	
Ban III	0	
Bbu I	3	2252, 3988, 4060
Bbv I	14	562, 953, 1256, 1462, 1465, 1555, 1974, 1992, 2072, 3448, 3804, 4248, 4451, 4548
Bcl I	0	
Bgl I	2	878, 4318
Bgl II	0	
Bsm I	5	2521, 2928, 3250, 3767, 3860
Bsp1286 I	5	328, 413, 1574, 2999, 4389
BspH I	3	51, 156, 1164
BspM I	1	2247
BspM II	1	2524
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	17	105, 437, 774, 867, 1260, 1369, 1543, 1643, 1710, 1980, 2013, 2040, 2105, 4330, 4469, 4499, 4602
Cfr I	4	603, 2044, 2793, 4174
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	12	69, 495, 1035, 1201, 1610, 2276, 2301, 2749, 3135, 3274, 3386, 4392
Dpn I	18	315, 351, 368, 626, 672, 690, 1031, 1136, 1148, 1226, 1234, 1245, 1320, 2274, 3065, 3675, 3912, 4158, 4288
Dra I	7	418, 1110, 1129, 2448, 2787, 3128, 3718
Dra II	1	16
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	2	2528, 4166
EcoR V	0	
Fnu4H I	22	252, 481, 576, 603, 942, 1270, 1476, 1479, 1544, 1687, 1842, 1960, 1963, 1981, 2061, 2870, 3462, 3793, 4237, 4414, 4465, 4562
Fok I	8	557, 844, 1025, 2508, 3054, 4091, 4228, 4511
Fsp I	1	773
Hae II	3	1644, 2014, 4331
Hae III	15	18, 605, 872, 952, 1410, 1844, 1862, 1873, 2046, 2441, 2486, 2708, 2795, 4176, 4278
Hga I	4	464, 1194, 1772, 4503
HgiA I	4	328, 413, 1574, 4389
Hha I	17	105, 437, 774, 867, 1260, 1369, 1543, 1643, 1710, 1980, 2013, 2040, 2105, 4330, 4469, 4499, 4602
Hinc II	2	2262, 3779
Hind II	2	2262, 3779
Hind III	1	2242
Hinf I	11	997, 1514, 1910, 1985, 2049, 2263, 2878, 3181, 3205, 3314, 3397
Hpa I	1	3779
Hpa II	17	459, 701, 811, 878, 912, 1316, 1506, 1532, 1679, 2167, 2483, 2525, 2653, 3061, 4163, 4521, 4555
Hph I	14	269, 304, 510, 926, 1153, 2564, 2570, 2572, 2708, 2764, 2776, 2818, 4573, 4582
Kpn I	0	
Mae I	7	803, 1138, 1391, 2267, 3291, 3669, 3865
Mae II	8	74, 394, 767, 1183, 2615, 2790, 4190, 4448
Mae III	18	335, 523, 676, 734, 1065, 1348, 1464, 1527, 2122, 2585, 2690, 3094, 3499, 3804, 4194, 4124, 4449, 4544
Mbo I	19	313, 349, 366, 624, 670, 688, 1029, 1134, 1146, 1224, 1232, 1243, 1318, 2272, 3063, 3673, 3910, 4156, 4286
Mbo II	12	18, 214, 323, 401, 1156, 1227, 2018, 2803, 3290, 3355, 3368, 4284
Mlu I	0	
Mnl I	22	29, 622, 828, 976, 1057, 1457, 1706, 1781, 1989, 2039, 2137, 2262, 2373, 3249, 3294, 3342, 3694, 3735, 3744, 4267, 4566, 4596
Msp I	17	459, 701, 811, 878, 912, 1316, 1506, 1532, 1679, 2167, 2483, 2525, 2653, 3061, 4163, 4521, 4555
Nae I	0	
Nar I	1	4328
Nci I	6	460, 811, 1507, 4163, 4521, 4556
Nco I	2	2829, 4147
Nde I	1	4380
Nhe I	0	
Nla III	21	55, 160, 553, 589, 667, 677, 1168, 1888, 2233, 2252, 2603, 2833, 2894, 2915, 3404, 3906, 3988, 4060, 4151, 4463, 4568
Nla IV	13	109, 699, 910, 951, 1045, 1817, 1856, 2141, 2996, 3078, 3958, 4030, 4329
Not I	0	
Nsi I	2	3990, 4062
PflM I	2	2760, 3215
PpuM I	0	
Pst I	1	2258
Pvu I	2	627, 4289
Pvu II	3	2063, 2428, 4258
Rsa I	6	515, 2407, 2945, 3607, 4397, 4431
Sac I	0	
Sac II	0	
Sal I	1	2260
Sau3A I	19	313, 349, 366, 624, 670, 688, 1029, 1134, 1146, 1224, 1232, 1243, 1318, 2272, 3063, 3673, 3910, 4156, 4286
Sau96 I	6	16, 632, 854, 871, 950, 4277
Sca I	2	515, 2945
ScrF I	17	460, 811, 1507, 1725, 1738, 1859, 2146, 2705, 2761, 3009, 3954, 4009, 4026, 4163, 4210, 4521, 4556
SfaN I	13	295, 544, 735, 1787, 2371, 2856, 3832, 3997, 4069, 4324, 4364, 4400, 4533
Sfi I	0	
Sin I	2	632, 854
Sma I	0	
Spe I	0	
Sph I	3	2252, 3988, 4060
Spo I	0	
Ssp I	4	191, 2840, 3494, 3647
Sst I	0	
Sst II	0	
Stu I	0	
Sty I	3	2829, 3363, 4147
Taq I	3	342, 1786, 2261
Tha I	10	105, 437, 930, 1260, 1841, 2038, 2040, 4497, 4600, 4602
Tth111 I	1	4455
Xba I	1	2266
Xho I	0	
Xho II	10	349, 366, 1134, 1146, 1232, 1243, 2272, 3063, 3910, 4156
Xma I	0	
Xma III	0	
Xmn I	1	396

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pCAT®-Promoter

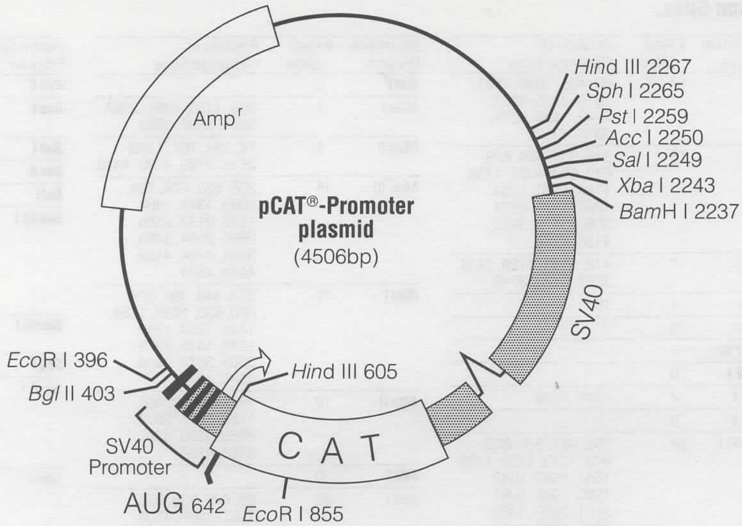


Figure 58. pCAT-Promoter vector circle map.

Notes:

1. Sequence reference points:
   a. multiple cloning sites (*Xba* I - *Hind* III) 2243-2267
   b. SV40 promoter region 408-610
   c. CAT gene start site 642
   d. CAT gene stop site 1301
   e. β-lactamase (Amp^r) coding region 4306-3446
2. Specialized application:
   a. analysis of eukaryotic enhancer sequences

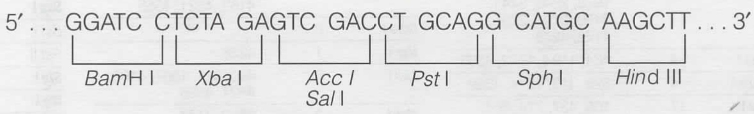


Figure 59. pCAT-Promoter plasmid multiple cloning sequence downstream of the SV40 sequence.

Table 33. pCAT-Promoter Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	4441
Acc I	1	2250
Acc III	1	851
Acy I	3	236, 4056, 4438
Aha II	3	236, 4056, 4438
Alu I	24	44, 63, 308, 553, 607, 627, 636, 755, 884, 1459, 1788, 1942, 2122, 2269, 2291, 2386, 2450, 2568, 2794, 2930, 3187, 3708, 3808, 3871
Alw44 I	3	177, 2940, 4186
Apa I	0	
Asu I	6	286, 3561, 3640, 3657, 3879, 4495
Asu II	0	See **Csp45 I**
Ava I	0	
Ava II	2	3657, 3879
Bal I	1	1122
BamH I	1	2237
Ban I	4	235, 1321, 2370, 3467
Ban II	0	
Ban III	0	
Bbu I	1	2265
Bbv I	14	53, 241, 314, 1775, 2131, 2437, 2518, 2536, 2955, 3045, 3048, 3254, 3557, 3948
Bcl I	0	
Bgl I	3	251, 542, 3639
Bgl II	1	403
Bsm I	5	848, 1255, 1577, 2094, 2187
Bsp1286 I	5	181, 1326, 2944, 4105, 4190
BspH I	3	3346, 4354, 4459
BspM I	1	2262
BspM II	1	851
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	17	5, 108, 238, 259, 2410, 2475, 2503, 2536, 2806, 2873, 2973, 3147, 3256, 3649, 3742, 4079, 4411
Cfr I	4	388, 1120, 2465, 3907
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	12	171, 549, 628, 1076, 1462, 1601, 1713, 2901, 3310, 3476, 4016, 4442
Dpn I	18	278, 405, 1392, 2002, 2239, 3194, 3269, 3280, 3288, 3366, 3378, 3483, 3824, 3842, 3888, 4146, 4163, 4199
Dra I	7	775, 1114, 1455, 2045, 3385, 3404, 4096
Dra II	1	4495
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	2	396, 855
EcoR V	0	
Fnu4H I	23	42, 151, 255, 328, 537, 1197, 1789, 2120, 2451, 2532, 2550, 2553, 2671, 2826, 2969, 3034, 3037, 3243, 3571, 3910, 3937, 4032, 4261
Fok I	8	90, 334, 440, 835, 1381, 3485, 3666, 3953
Fsp I	2	258, 3741
Hae II	3	239, 2504, 2874
Hae III	19	288, 390, 530, 536, 545, 588, 768, 813, 1035, 1122, 2467, 2641, 2652, 2670, 3104, 3562, 3642, 3909, 4496
Hga I	4	97, 2737, 3315, 4045
HgiA I	4	181, 2944, 4105, 4190
Hha I	17	5, 108, 238, 259, 2410, 2475, 2503, 2536, 2806, 2873, 2973, 3147, 3256, 3649, 3742, 4079, 4411
Hinc II	2	2106, 2251
Hind II	2	2106, 2251
Hind III	2	605, 2267
Hinf I	11	1205, 1508, 1532, 1641, 1724, 2247, 2461, 2526, 2601, 2997, 3514
Hpa I	1	2106
Hpa II	16	48, 82, 810, 852, 980, 1388, 2344, 2833, 2980, 3006, 3196, 3600, 3634, 3701, 3811, 4053
Hph I	14	24, 33, 891, 897, 899, 1035, 1091, 1103, 1145, 3362, 3589, 4005, 4211, 4246
Kpn I	0	
Mae I	8	590, 1618, 1996, 2192, 2244, 3121, 3374, 3709
Mae II	7	374, 942, 1117, 3329, 3745, 4118, 4438
Mae III	16	56, 347, 367, 912, 1017, 1421, 1826, 2131, 2982, 3045, 3161, 3444, 3775, 3833, 3986, 4174
Mbo I	18	276, 403, 1390, 2000, 2237, 3192, 3267, 3278, 3286, 3364, 3376, 3481, 3822, 3840, 3886, 4144, 4161, 4197
Mbo II	11	283, 1130, 1617, 1682, 1695, 2497, 3288, 3359, 4114, 4192, 4301
Mlu I	0	
Mnl I	27	39, 299, 519, 525, 550, 556, 562, 565, 577, 700, 1576, 1621, 1669, 2021, 2062, 2071, 2251, 2474, 2525, 2733, 2808, 3057, 3457, 3538, 3686, 3892, 4485
Msp I	16	48, 82, 810, 852, 980, 1388, 2344, 2833, 2980, 3006, 3196, 3600, 3634, 3701, 3811, 4053
Nae I	0	
Nar I	1	236
Nci I	5	48, 83, 3006, 3702, 4053
Nco I	2	496, 1156
Nde I	1	184
Nhe I	0	
Nla III	18	41, 500, 930, 1160, 1221, 1242, 1731, 2233, 2265, 2284, 2630, 3350, 3841, 3851, 3929, 3965, 4358, 4463
Nla IV	12	237, 1323, 1405, 2239, 2372, 2658, 2697, 3469, 3563, 3604, 3815, 4405
Not I	0	
Nsi I	0	
PflM I	2	1087, 1542
PpuM I	0	
Pst I	1	2259
Pvu I	2	279, 3889
Pvu II	3	308, 755, 2450
Rsa I	5	169, 734, 1272, 1934, 3999
Sac I	0	
Sac II	0	
Sal I	1	2249
Sau3A I	18	276, 403, 1390, 2000, 2237, 3192, 3267, 3278, 3286, 3364, 3376, 3481, 3822, 3840, 3886, 4144, 4161, 4197
Sau96 I	6	286, 3561, 3640, 3657, 3879, 4495
Sca I	2	1272, 3999
ScrF I	13	48, 83, 355, 1032, 1088, 1336, 2366, 2654, 2775, 2788, 3006, 3702, 4053
SfaN I	12	68, 162, 198, 238, 418, 698, 1183, 2159, 2723, 3775, 3966, 4215
Sfi I	1	542
Sin I	2	3657, 3879
Sma I	0	
Spe I	0	
Sph I	1	2265
Spo I	1	1245
Ssp I	4	1167, 1821, 1974, 4323
Sst I	0	
Sst II	0	
Stu I	1	588
Sty I	4	496, 589, 1156, 1690
Taq I	3	2250, 2726, 4170
Tha I	10	3, 5, 108, 2473, 2475, 2673, 3254, 3584, 4077, 4409
Tth111 I	0	
Xba I	1	2243
Xho I	0	
Xho II	9	403, 1390, 2237, 3267, 3278, 3364, 3376, 4144, 4161
Xma I	0	
Xma III	0	
Xmn I	1	4118

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pCAT®-Control

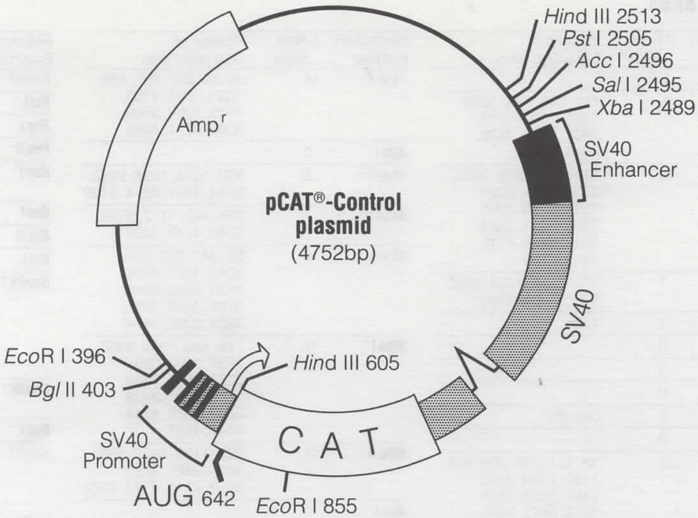


Figure 60. pCAT-Control vector circle map.

Notes:

1. Sequence reference points:
   a. multiple cloning sites (*Xba* I - *Pst* I) 2494-2506
   b. SV40 promoter region 408-610
   c. SV40 enhancer region 2243-2479
   d. CAT gene start site 642
   e. CAT gene stop site 1301
   f. β-lactamase (Amp^r) coding region 4552-3692
2. Specialized application:
   a. control for transfection efficiency

Technical Services:
800-356-9526

Table 34. pCAT -Control Vector Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	1	4685
Acc I	1	2496
Acc III	1	851
Acy I	3	236, 4300, 4682
Aha II	3	236, 4300, 4682
Alu I	24	44, 63, 308, 553, 607, 627, 636, 755, 884, 1459, 1788, 1942, 2122, 2515, 2537, 2632, 2696, 2814, 3040, 3176, 3432, 3952, 4052, 4115
Alw44 I	3	177, 3186, 4430
Apa I	0	
Asu I	6	286, 3805, 3884, 3901, 4123, 4739
Asu II	0	See **Csp45 I**
Ava I	0	
Ava II	2	3901, 4123
Bal I	1	1122
BamH I	0	
Ban I	4	235, 1321, 2616, 3711
Ban II	0	
Ban III	0	
Bbu I	3	2315, 2387, 2511
Bbv I	14	53, 241, 314, 1775, 2131, 2683, 2764, 2782, 3201, 3291, 3294, 3498, 3801, 4192
Bcl I	0	
Bgl I	3	251, 542, 3883
Bgl II	1	403
Bsm I	5	848, 1255, 1577, 2094, 2187
Bsp1286 I	5	181, 1326, 3190, 4349, 4434
BspH I	3	3590, 4598, 4703
BspM I	1	2508
BspM II	1	851
BssH II	0	
BstE II	0	
BstX I	0	
Bsu36 I	0	
Cfo I	17	5, 108, 238, 259, 2656, 2721, 2749, 2782, 3052, 3119, 3219, 3392, 3500, 3893, 3986, 4323, 4655
Cfr I	4	388, 1120, 2711, 4151
Cla I	0	
Csp I	0	
Csp45 I	0	
Dde I	13	171, 549, 628, 1076, 1462, 1601, 1713, 3147, 3328, 3554, 3720, 4260, 4686
Dpn I	19	278, 405, 1392, 2002, 2239, 2485, 3439, 3513, 3524, 3532, 3610, 3622, 3727, 4068, 4086, 4132, 4390, 4407, 4443
Dra I	7	775, 1114, 1455, 2045, 3629, 3648, 4340
Dra II	1	4739
Dra III	0	
Eco47 III	0	
Eco52 I	0	
EcoR I	2	396, 855
EcoR V	0	
Fnu4H I	23	42, 151, 255, 328, 537, 1197, 1789, 2120, 2697, 2778, 2796, 2799, 2917, 3072, 3215, 3280, 3283, 3487, 3815, 4154, 4181, 4276, 4505
Fok I	9	90, 334, 440, 835, 1381, 2418, 3729, 3910, 4197
Fsp I	2	258, 3985
Hae II	3	239, 2750, 3120
Hae III	19	288, 390, 530, 536, 545, 588, 768, 813, 1035, 1122, 2713, 2887, 2898, 2916, 3349, 3806, 3886, 4153, 4740
Hga I	4	97, 2983, 3559, 4289
HgiA I	4	181, 3190, 4349, 4434
Hha I	17	5, 108, 238, 259, 2656, 2721, 2749, 2782, 3052, 3119, 3219, 3392, 3500, 3893, 3986, 4323, 4655
Hinc II	2	2106, 2497
Hind II	2	2106, 2497
Hind III	2	605, 2513
Hinf I	11	1205, 1508, 1532, 1641, 1724, 2493, 2707, 2772, 2847, 3243, 3758
Hpa I	1	106
Hpa II	16	48, 82, 810, 852, 980, 1388, 2590, 3079, 3226, 3252, 3441, 3844, 3878, 3945, 4055, 4297
Hph I	14	24, 33, 891, 897, 899, 1035, 1091, 1103, 1145, 3606, 3833, 4249, 4455, 4490
Kpn I	0	
Mae I	8	590, 1618, 1996, 2192, 2490, 3366, 3618, 3953
Mae II	7	374, 942, 1117, 3573, 3989, 4362, 4682
Mae III	16	56, 347, 367, 912, 1017, 1421, 1826, 2131, 3228, 3291, 3406, 3688, 4019, 4077, 4230, 4418
Mbo I	19	276, 403, 1390, 2000, 2237, 2483, 3437, 3511, 3522, 3530, 3608, 3620, 3725, 4066, 4084, 4130, 4388, 4405, 4441
Mbo II	10	283, 1130, 1617, 1682, 1695, 2743, 3605, 4360, 4438, 4547
Mlu I	0	
Mnl I	27	39, 299, 519, 525, 550, 556, 562, 565, 577, 700, 1576, 1621, 1669, 2021, 2062, 2071, 2497, 2720, 2771, 2979, 3054, 3303, 3703, 3784, 3932, 4138, 4731
Msp I	16	48, 82, 810, 852, 980, 1388, 2590, 3079, 3226, 3252, 3441, 3844, 3878, 3945, 4055, 4297
Nae I	0	
Nar I	1	236
Nci I	5	48, 83, 3252, 3946, 4297
Nco I	3	496, 1156, 2474
Nde I	1	184
Nhe I	0	
Nla III	21	41, 500, 930, 1160, 1221, 1242, 1731, 2233, 2315, 2387, 2478, 2511, 2530, 2876, 3594, 4085, 4095, 4173, 4209, 4602, 4707
Nla IV	13	237, 1323, 1405, 2285, 2357, 2618, 2904, 2943, 3713, 3807, 3848, 4059, 4649
Not I	0	
Nsi I	2	2317, 2389
PflM I	2	1087, 1542
PpuM I	0	
Pst I	1	2505
Pvu I	2	279, 4133
Pvu II	3	308, 755, 2696
Rsa I	5	169, 734, 1272, 1934, 4243
Sac I	0	
Sac II	0	
Sal I	1	2495
Sau3A I	19	276, 403, 1390, 2000, 2237, 2483, 3437, 3511, 3522, 3530, 3608, 3620, 3725, 4066, 4084, 4130, 4388, 4405, 4441
Sau96 I	6	286, 3805, 3884, 3901, 4123, 4739
Sca I	2	1272, 4243
ScrF I	16	48, 83, 355, 1032, 1088, 1336, 2281, 2366, 2353, 2612, 2900, 3021, 3034, 3252, 3946, 4297
SfaN I	14	68, 162, 198, 238, 418, 698, 1183, 2159, 2324, 2396, 2969, 4019, 4210, 4459
Sfi I	1	542
Sin I	2	3901, 4123
Sma I	0	
Spe I	0	
Sph I	3	2315, 2387, 2511
Spo I	0	
Ssp I	4	1167, 1821, 1974, 4567
Sst I	0	
Sst II	0	
Stu I	1	588
Sty I	5	496, 589, 1156, 1690, 2474
Taq I	3	2496, 2972, 4414
Tha I	10	3, 5, 108, 2719, 2721, 2919, 3498, 3828, 4321, 4653
Tth111 I	0	
Xba I	1	2489
Xho I	0	
Xho II	10	403, 1390, 2237, 2483, 3511, 3522, 3608, 3620, 4388, 4405
Xma I	0	
Xma III	0	
Xmn I	1	4362

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

pSV-β-Galactosidase

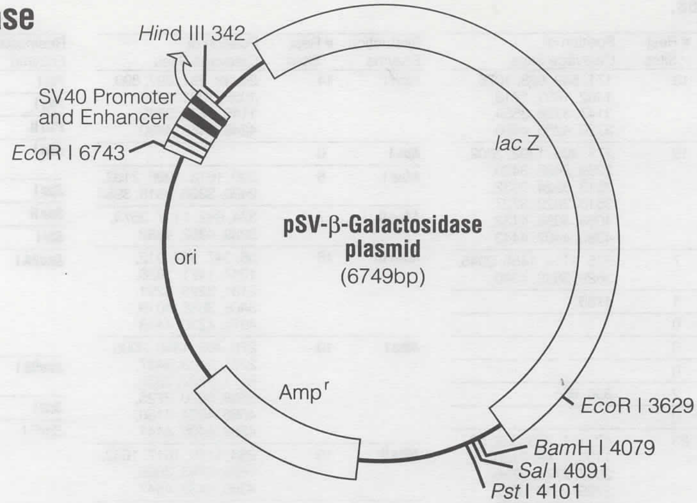


Figure 61. pSV-β-Galactosidase circle map. The arrow indicates the direction of transcription of the *lacZ* gene.

Notes:

1. Sequence reference points:
   a. lac operon sequences 637-3948
   b. β-lactamase (Amp^r) region 4712-5572
   c. SV40 promoter and enhancer region
2. Specialized applications:
   a. control for transfection efficiency
3. *Eco*R I cuts in two postions in the pSV-β-galactosidase vector.

Table 35. pSV-β-Galactosidase Restriction Sites.

Restriction Enzyme	# Rest. Sites	Position of Cleavage Sites
Aat II	2	1248, 4580
Acc I	2	3389, 4092
Acc III	0	
Acy I	0	
Afl II	0	
Aha II	0	
Alu I	26	74, 290, 344, 717, 828, 1008, 2563, 2863, 3274, 3637, 3964, 4111, 4203, 4448, 4467, 5146, 5209, 5309, 5830, 6087, 6133, 6223, 6449, 6567, 6631, 6726
Alw44 I	5	2970, 3085, 4330, 4827, 6073
Apa I	0	
ApaL I	5	2970, 3085, 4330, 4827, 6073
Asu I	11	736, 2166, 3052, 3314, 3452, 4222, 4519, 5135, 5357, 5374, 5483
Ava I	2	1963, 3406
Ava II	3	2166, 5135, 5357
Bal I	0	
BamH I	1	4079
Ban I	9	48, 547, 805, 817, 1421, 1873, 4272, 5546, 6643
Ban II	2	2565, 3614
Ban III	2	630, 1449
Bbu I	2	1855, 4694
Bbv I	29	15, 707, 780, 1164, 1242, 1623, 1626, 1957, 2494, 2683, 2776, 2937, 3083, 3151, 3413, 3525, 3973, 4193, 4266, 4454, 5065, 5456, 5759, 5965, 5968, 6058, 6477, 6495, 6576
Bcl I	1	1971
Bgl I	5	279, 777, 2898, 4263, 5381
Bgl II	0	
Bsm I	1	4029
Bsp1286 I	9	2565, 2751, 2974, 3089, 3614, 4334, 4831, 4916, 6077
BspM I	3	1399, 3271, 4104
BspM II	0	
BssH II	1	2123
BstE II	0	
BstN I	15	110, 449, 667, 794, 1098, 2191, 2759, 3026, 3060, 3605, 4153, 6226, 6239, 6360, 6648,
BstX I	2	2845, 3462
Cfo I	48	3, 367, 616, 768, 789, 904, 1083, 1136, 1148, 1325, 1429, 1520, 1776, 1905, 1950, 2009, 2023, 2125, 2127, 2205, 2461, 2584, 2684, 2759, 2955, 3253, 3280, 3426, 3438, 3468, 3644, 4254, 4275,
Cfo I (cont.)		4405, 4508, 4608, 4940, 5277, 5370, 5763, 5872, 6046, 6146, 6213, 6483, 6516, 6544, 6609
Cla I	2	630, 1449
Csp I	0	
Dde I	13	56, 286, 584, 849, 1130, 2789, 3638, 4337, 4572, 4998, 5538, 5704, 6113
Dpn I	33	351, 467, 629, 633, 747, 843, 1209, 1473, 1899, 1953, 1973, 2036, 2049, 2213, 2328, 2406, 2958, 3369, 4081, 4233, 4818, 4854, 4871, 5129, 5175, 5193, 5534, 5639, 5651, 5729, 5737, 5748, 5823
Dra I	4	3747, 4921, 5613, 5632
Dra II	1	4519
Dra III	1	1815
Eco47 III	1	2460
Eco52 I	0	
EcoR I	2	3629, 6743
EcoR V	1	1738
Fnu4H I	54	4, 274, 589, 696, 769, 1178, 1231, 1256, 1321, 1354, 1420, 1637, 1640, 1946, 2077, 2305, 2450, 2483, 2597, 2672, 2765, 2808, 2948, 2951, 3048, 3072, 3140, 3317, 3348, 3427, 3469, 3493, 3539, 3885, 3962, 4182, 4255, 4359, 4468, 4755, 4984, 5079, 5106, 5445, 5773, 5979, 5982, 6047, 6190, 6345, 6463, 6466, 6484, 6565
Fok I	20	177, 687, 1237, 1677, 1727, 1782, 1856, 2144, 2439, 2587, 2600, 2636, 2807, 3231, 3778, 4173, 4417, 5060, 5347, 5528
Hae I	9	14, 325, 1023, 2001, 3261, 3700, 5913, 6365, 6376
Hae II	12	790, 1084, 1521, 2010, 2462, 2585, 2685, 2760, 3645, 4276, 6147, 6517
Hae III	29	14, 267, 273, 282, 325, 737, 854, 1023, 1098, 1830, 2001, 2096, 3054, 3065, 3261, 3316, 3453, 3700, 4121, 4223, 4521, 5108, 5375, 5455, 5913, 6347, 6365, 6376, 6550
Hga I	13	1036, 1465, 2027, 2209, 2762, 2799, 2806, 2953, 3442, 4409, 4967, 5697, 6275
HgiA I	7	2565, 2974, 3089, 4334, 4831, 4916, 6077
Hha I	48	3, 367, 616, 768, 789, 904, 1083, 1136, 1148, 1325, 1429, 1520, 1776, 1905, 1950, 2009, 2023, 2125, 2127, 2205, 2461, 2584, 2684, 2759, 2955,
Hha I (cont.)		3253, 3280, 3426, 3438, 3468, 3644, 4254, 4275, 4405, 4508, 4608, 4940, 5277, 5370, 5763, 5872, 6046, 6146, 6213, 6483, 6516, 6544, 6609
Hinc II	4	1051, 1675, 3503, 4093
Hind III	1	342
Hinf I	12	964, 1531, 1663, 1882, 1994, 3602, 4089, 5500, 6017, 6413, 6488, 6553
Hpa I	2	1051, 1675
Hph I	16	439, 569, 1180, 1412, 1451, 1952, 2393, 2621, 2956, 4479, 4488, 4772, 4807, 5013, 5429, 5656
Kpn I	2	52, 551
Mae I	6	327, 4034, 4086, 5306, 5641, 5894
Mae II	12	475, 649, 918, 1245, 1486, 1494, 3399, 4135, 4577, 4897, 5270, 5686
Mae III	29	445, 653, 673, 893, 919, 977, 1091, 1187, 1241, 1364, 1378, 1477, 1679, 1930, 2240, 2524, 2794, 3973, 4139, 4159, 4400, 4838, 5026, 5179, 5237, 5568, 5851, 5967, 6030
Mbo I	?	?
Mbo II	14	572, 743, 836, 1217, 2147, 3394, 3560, 4229, 4717, 4826, 4904, 5659, 5730, 6521
Mlu I	3	1927, 2707, 3132
Mnl I	35	26, 35, 50, 256, 262, 287, 293, 299, 302, 314, 391, 587, 726, 843, 883, 1172, 1326, 1605, 1660, 1704, 2628, 2798, 4093, 4212, 4472, 4532, 5125, 5331, 5479, 5560, 5960, 6209, 6284, 6492, 6543
Msp I	2	1051, 1675
Nae I	0	
Nar I	1	4273
Nci I	11	2060, 2150, 2509, 2686, 2746, 3693, 4427, 4462, 4963, 5314, 6010
Nco I	2	9, 233
Nde I	2	3582, 4325
Nhe I	0	
Nla III	28	13, 146, 237, 430, 460, 473, 1292, 1703, 1714, 1873, 2736, 3388, 3561, 3705, 3805, 3842, 4075, 4107, 4474, 4558, 4663, 5056, 5092, 5770, 5180, 5671, 6391, 6737
Nla IV	20	50, 116, 549, 807, 819, 1423, 1875, 2083, 3611, 3808, 4081, 4274, 4612, 5202, 5413, 5454, 5548, 6320, 6359, 6645
Not I	0	
Nru I	0	
Nsi I	1	148
PflM I	2	1870, 2236
Pst I	1	4101
Pvu I	6	748, 1474, 1954, 2407, 4234, 5130
Pvu II	6	74, 717, 3274, 3637, 4203, 6567
Rsa I	10	50, 549, 1329, 1808, 2120, 2706, 3398, 3740, 4342, 5018
Sac I	1	2565
Sac II	0	
Sal I	1	4091
Sau3A I	I	349, 465, 627, 631, 745, 841, 1207, 1471, 1897, 1951, 1971, 2034, 2047, 2211, 2326, 2404, 2956, 3367, 4079, 4231, 4816, 4852, 4869, 5127, 5173, 5191, 5532, 5637, 5649, 5727, 5735, 5746, 5821
Sau96 I	11	736, 2166, 3052, 3314, 3452, 4222, 4519, 5135, 5357, 5374, 5453
Sca I	1	5018
ScrF I	26	112, 451, 669, 796, 1100, 2060, 2150, 2193, 2509, 2686, 2746, 2761, 3028, 3062, 3607, 3693, 4155, 4427, 4462, 4963, 5314, 6010, 6228, 6241, 6362, 6650
SfaN I	20	155, 486, 876, 889, 1696, 2461, 2836, 2865, 3097, 3151, 3253, 4001, 4269, 4309, 4345, 4439, 4798, 5047, 5238, 6290
Sfi I	1	279
Sin I	3	2166, 5135, 5357
Sma I	0	
SnaB I	0	
Spe I	0	
Sph I	2	146, 4107
Spo I	0	
Ssp I	2	1855, 4694
Sst I	1	2565
Sst II	0	
Stu I	1	325
Sty I	3	9, 233, 326
Taq I	14	383, 579, 630, 1449, 1497, 1666, 2046, 2520, 2859, 3033, 3570, 4092, 4845, 6289
Tth111 II	4	3911, 5764, 5796, 5803
Xba I	1	4085
Xho I	0	

Note:
The enzymes listed in boldface type are manufactured by Promega.

Vector Maps

EMBL3 and EMBL4

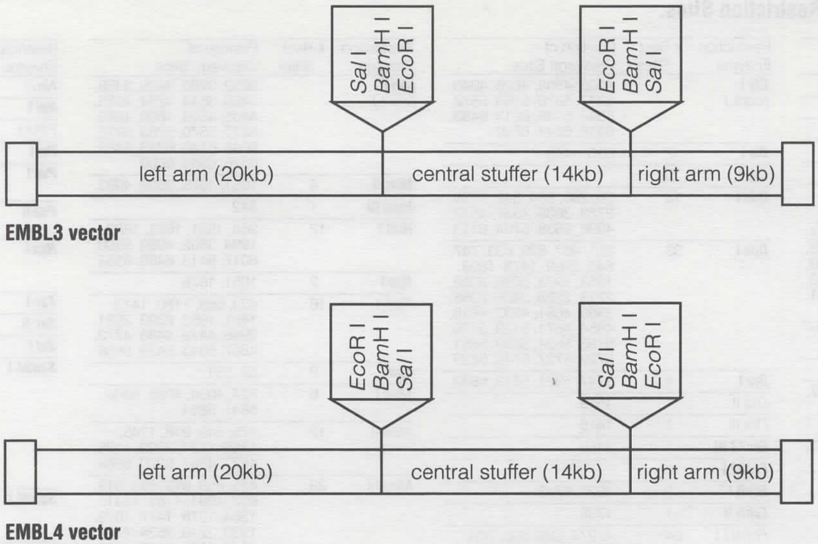


Figure 62. Schematic diagrams of the EMBL3 and EMBL4 lambda cloning vectors.

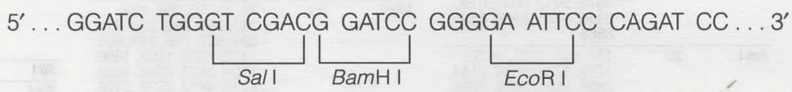


Figure 63. EMBL3 and EMBL4 vector polylinker sequence. The polylinker sequence is present in opposite orientations in the two vectors.

Table 36. EMBL3 and EMBL4 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
*Bam*H I	2	19.40, 33.10
*Eco*R I	2	19.40, 33.10
*Hin*d III	3	26.90, 32.60, 37.90
Sal I	2	19.40, 33.10

Notes:

1. The EMBL3 and EMBL4 genomic cloning vectors have a cloning capacity of 9-23kb and allow *Spi* selection for recombinants.
2. The EMBL3 and EMBL4 vectors differ only in the orientations of the polylinker regions.

References:

1. Frischauf, A., Lehrach, H., Poustka, A., and Murray., N. (1983) J. *Mol. Biol.* **170**, 827.
2. Kern, J.M., *et al.* (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5172-5176.

LambdaGEM®-11

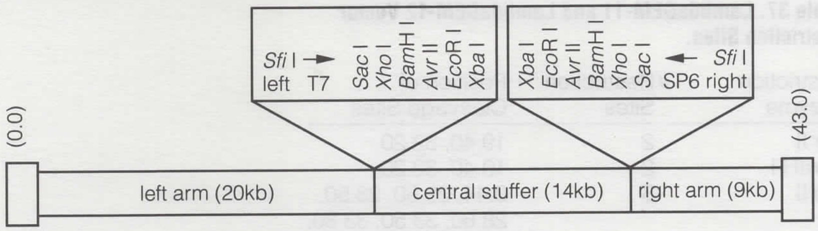


Figure 64. Structural map of the LambdaGEM-11 vector.

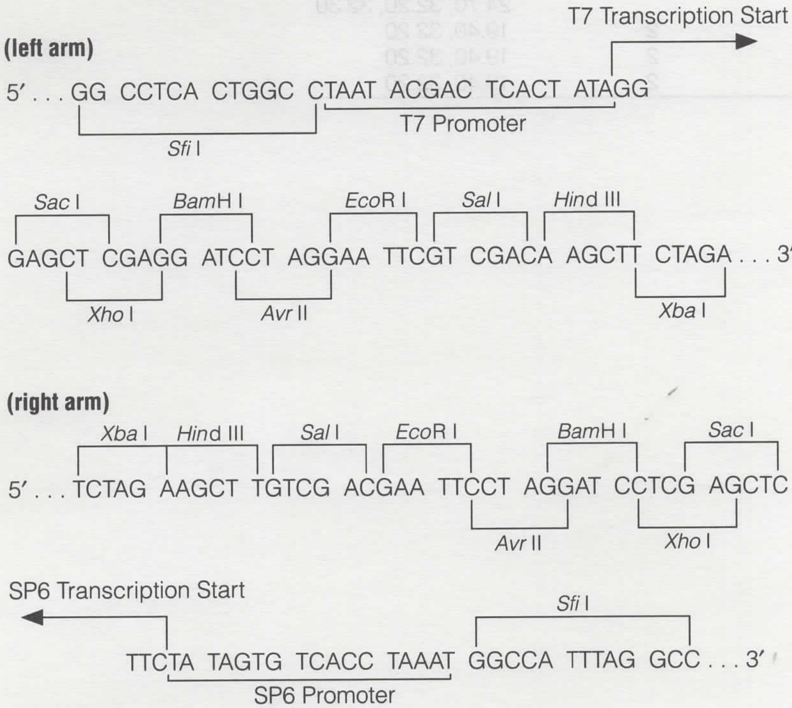


Figure 65. LambdaGEM-11 vector promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

Notes:

1. The LambdaGEM-11 genomic cloning vector is designed for high resolution restriction mapping of inserts, genomic walking, and simplified genomic library construction.
2. The LambdaGEM-11 arms are derived from EMBL3 (1) and the stuffer fragment is derived from lambda 2001 (2).

References:

1. Frischauf, A., Lehrach, H., Poustka, A., and Murray, N. (1983) *J. Mol. Biol.* **170**, 827.
2. Karn, J. (1984) *Gene* **32**, 217.

LambdaGEM®-11

Table 37. LambdaGEM-11 and LambdaGEM-12 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
Avr II	2	19.40, 32.20
*Bam*H I	2	19.40, 32.20
Bgl II	8	0.44, 25.60, 28.50, 28.60, 33.50, 33.80, 34.50, 34.60
*Eco*R I	2	19.40, 32.20
*Hin*d III	3	19.40, 32.20, 37.10
Kpn I	2	17.10, 18.60
Sac I	2	19.40, 32.20
Sal I	6	19.30, 19.40, 22.30, 24.70, 32.20, 32.30
Sfi I	2	19.40, 32.20
Xba I	2	19.40, 32.20
Xho I	2	19.40, 32.20

LambdaGEM®-12

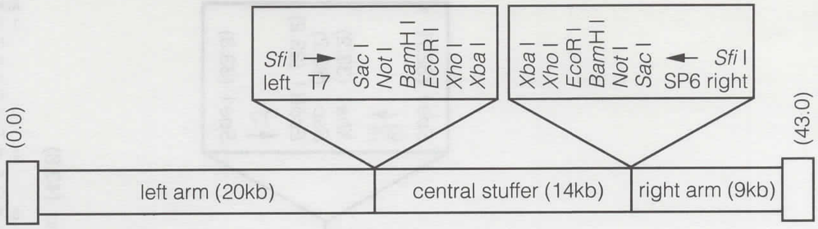


Figure 66. Structural map of the LambdaGEM-12 vector.

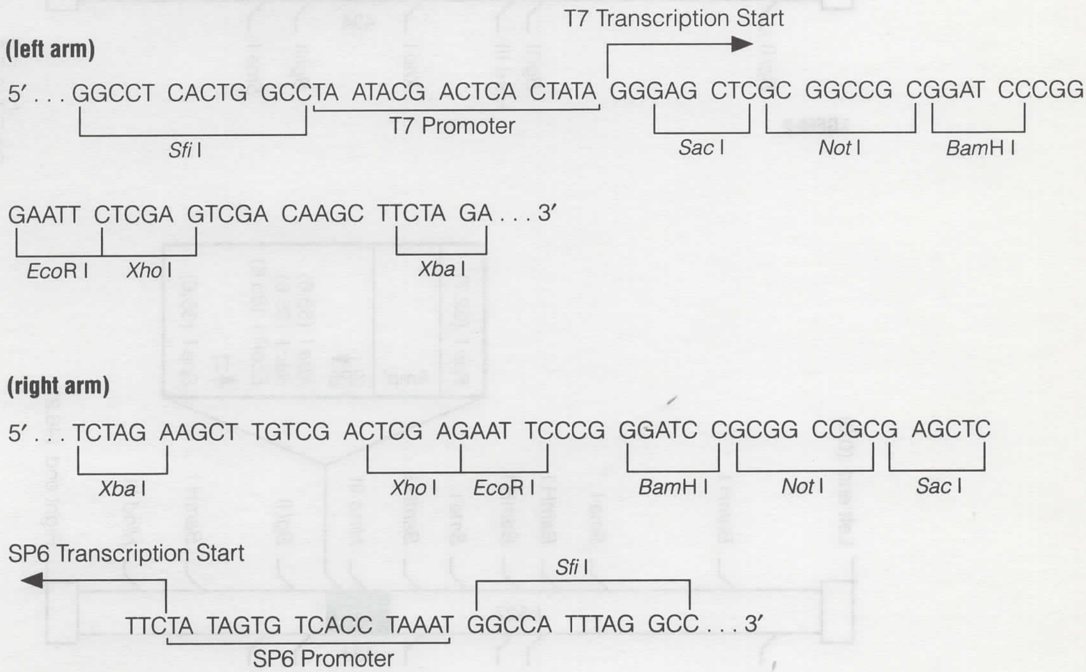


Figure 67. LambdaGEM-12 vector promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by T7 RNA polymerase and is complementary to RNA synthesized by SP6 RNA polymerase.

Note:

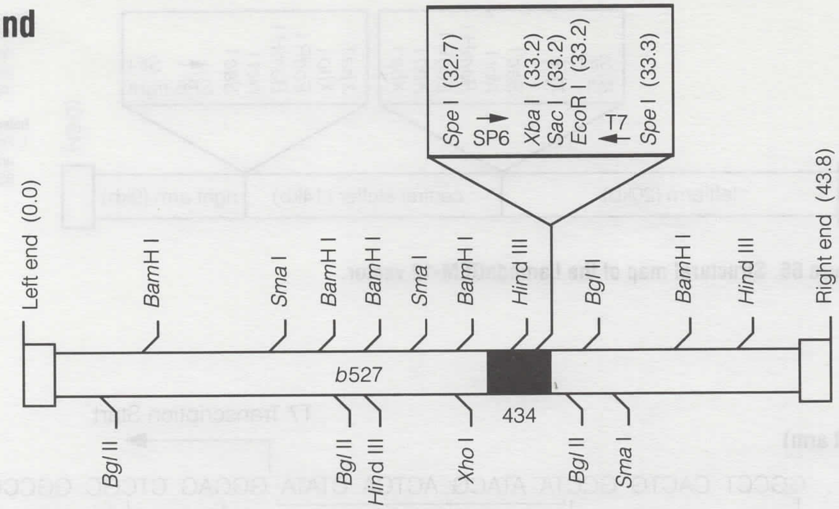
1. The LambdaGEM-12 vector is similar to the LambdaGEM-11 vector except that it contains *Not* I sites in the multiple cloning region and lacks *Avr* II sites.

Reference:

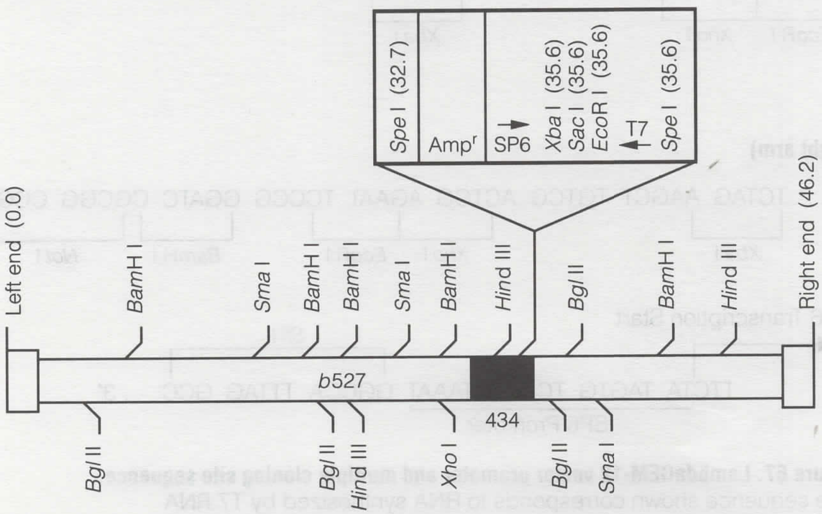
1. Frischauf, A., Lehrach, H., Poustka, A., and Murray, N. (1983) *J. Mol. Biol.* **170**, 827.

Vector Maps

LambdaGEM®-2 and LambdaGEM®-4



λGEM-2



λGEM-4

Figure 68. Structural maps of the LambdaGEM-2 and LambdaGEM-4 vectors.

Notes:

1. The LambdaGEM-2 and -4 vectors allow highly efficient, directional cDNA cloning and selective amplification of either sense or antisense cDNA sequences. Both vectors contain *Spe* I sites flanking the promoter and multiple cloning regions, greatly facilitating subcloning.
2. The LambdaGEM-2 and LambdaGEM-4 vector arms (1) are derived from lambda gt10 (2).
3. The LambdaGEM-2 vector contains a partial copy of the pGEM-1 plasmid, including the SP6 and T7 promoters, on either side of the multiple cloning region.
4. The LambdaGEM-4 vector contains a complete copy of the pGEM-1 plasmid.
5. LambdaGEM-2 cloning capacity: 0-7.1kb
6. LambdaGEM-4 cloning capacity: 0-4.1kb

References:

1. Palazzolo, M. and Meyerowitz, E. (1987) *Gene* **52**, 197.
2. Huynh, T., Young, R., and Davis, R. (1985) in: *DNA Cloning: A Practical Approach* (ed. D. Glover) IRL Press, Oxford, 49-78.

LambdaGEM®-2 and LambdaGEM®-4

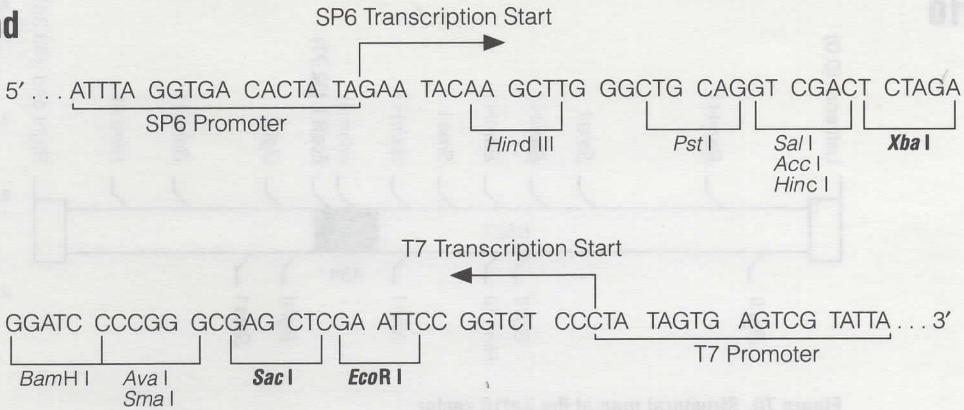


Figure 69. LambdaGEM-2 and LambdaGEM-4 vectors: promoter and multiple cloning site sequence. The sequence shown corresponds to RNA synthesized by SP6 RNA polymerase and is complementary to RNA synthesized by T7 RNA polymerase. The restriction sites which are listed in boldface are unique to both LambdaGEM-2 and LambdaGEM-4 vectors.

Table 38. LambdaGEM-2 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
*Bam*H I	6	5.50, 22.40, 24.00, 30.50, 33.20, 37.10
Bgl II	4	0.45, 22.50, 34.10, 34.20
*Eco*R I	1	33.20
*Hin*d III	4	23.20, 32.50, 33.20, 39.50
Sac I	1	33.20
Sma I	4	19.40, 27.60, 32.20, 35.30
Spe I	2	32.70, 33.30
Xba I	1	33.20
Xho I	1	29.50

Table 39. LambdaGEM-4 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
*Bam*H I	6	5.50, 22.40, 24.00, 30.50, 35.50, 39.50
Bgl II	4	0.45, 22.50, 36.50, 36.60
*Eco*R I	1	35.60
*Hin*d III	4	23.20, 32.50, 35.50, 41.90
Sac I	1	35.60
Sma I	4	19.40, 27.60, 35.60, 37.60
Spe I	2	32.70, 35.60
Xba I	1	35.60
Xho I	1	29.50

Vector Maps

Lambda gt10

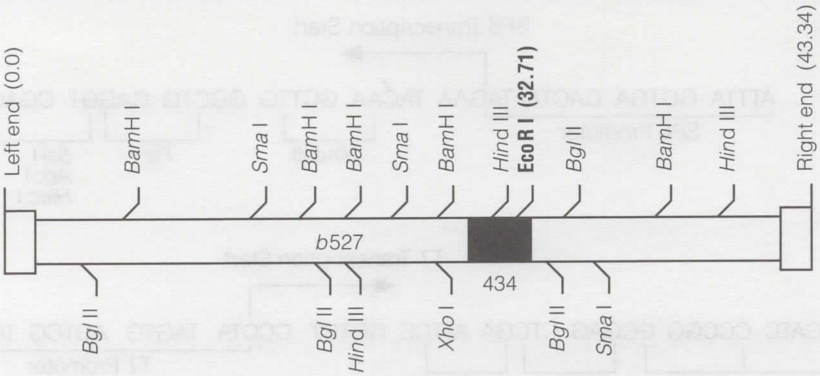


Figure 70. Structural map of the λgt10 vector.

5′ . . . TTAAG TCCAA GCTGA ATTCT TTTGC TTTTT . . . 3′

EcoR I

Figure 71. Lambda gt10 vector: sequence near the unique *EcoR* I restriction site.

Notes:

1. The lambda gt10 insertion vector is used for the construction of cDNA and genonic libraries (1). Phage libraries cloned into lambda gt10 are useful for screening with nucleic acid probes.
2. Lambda gt10 contains a unique *EcoR* I cloning site within the cI phage repressor gene.

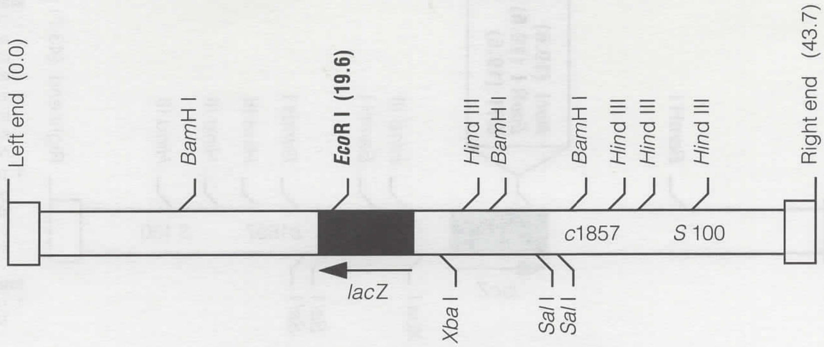
References:

1. Huynh, T.V., Young, R.A. and Davis, R.W. (1985) in: *DNA Cloning: A Practical Approach* (ed. D. Glover) IRL Press, Oxford, 49-78.
2. Murray, N.E., *et al.* (1977) *Mol. Gen. Genet.* **150**, 53.

Table 40. Lambda gt10 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
BamH	5	I5.5.50, 22.35, 23.97, 30.50, 36.59
Bgl II	4	0.42, 22.43, 33.61, 33.67
EcoR I	1	32.71
Hind III	3	23.13, 32.47, 39.00
Sma I	3	19.40, 27.62, 34.74
Xho I	1	29.50

Lambda gt11



Notes:

1. The Lambda gt11 cloning and expression vector (1) is used in constructing cDNA and genomic libraries. The unique *Eco*R I cloning site is located within the *lacZ* gene, upstream from the β-galactosidase translation termination codon.
2. The direction of transcription of the *lacZ* gene is indicated by the arrow. Also shown are the temperature-sensitive *c*I repressor, *c*1857, and the S amber mutation, S100.

Reference:

1. Young, R. and Davis, R., (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1194.

Figure 72. Structural map of the Lambda gt11 vector.

3′ . . . GTC GAC $\underbrace{\text{CTT AAG}}_{EcoR\ I}$ GCG GCT ATG . . . 5′

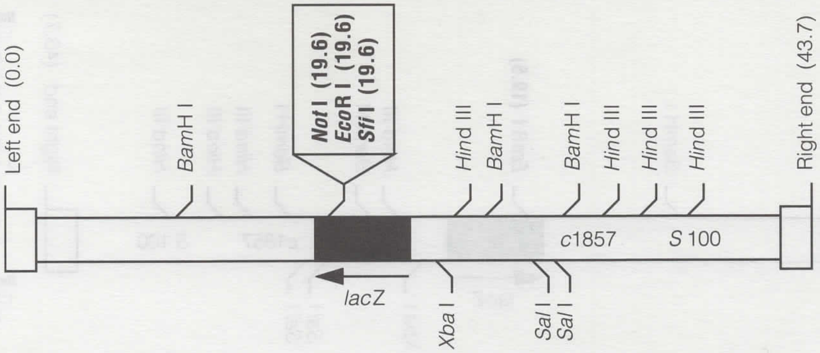
Figure 73. Lambda gt11 vector: in-frame sequence near the unique *Eco*R I restriction site.

Table 41. Lambda gt11 Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
*Bam*H I	3	5.51, 25.97, 32.50
Bgl II	5	0.42, 33.71, 36.10, 36.76, 36.82
*Eco*R I	1	19.60
Hind III	5	25.48, 34.90, 35.46, 35.58, 39.35
Kpn I	2	17.05, 18.56
Pvu I	6	11.93, 20.82, 21.35, 21.75, 22.47, 33.79
Sac I	2	20.66, 25.09
Sal I	2	30.75, 31.24
Xba I	1	24.82
Xho I	1	31.50

Vector Maps

Lambda gt11 *Sfi-Not*



Notes:

1. The Lambda gt11 *Sfi-Not* vector is designed for orientation-specific cDNA cloning and expression of cloned inserts as β-galactosidase fusion proteins.
2. The Lambda gt11 *Sfi-Not* vector is derived from the Lambda gt11 vector, with a synthetic polylinker containing unique *Not* I, *Eco*R I, and *Sfi* I restriction sites.
3. The direction of transcription of the *lac*Z gene is indicated by the arrow. Also shown are the temperature-sensitive cI repressor, *c*I857, and the S amber mutation, *S*100.

Reference:

1. Young, R. and Davis, R., (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1194.

Figure 74. Structural map of the Lambda gt11 *Sfi-Not* vector.

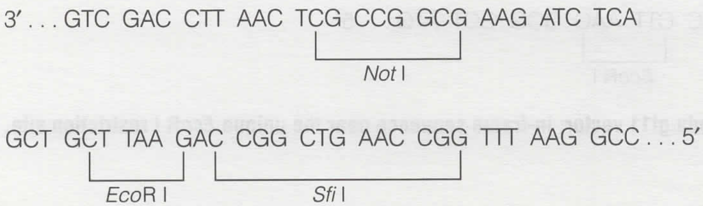


Figure 75. Lambda gt11 *Sfi-Not* vector: In-frame sequence and location of the unique restriction sites within the multiple cloning region.

Table 42. Lambda gt11 *Sfi-Not* Vector Restriction Sites.

Restriction Enzyme	# Restriction Sites	Position of Cleavage Sites
*Bam*H I	3	5.51, 25.97, 32.50
Bgl II	5	0.42, 33.71, 36.10, 36.76, 36.82
*Eco*R I	1	19.60
*Hin*d III	5	25.48, 34.90, 35.46, 35.58, 39.35
Kpn I	2	17.05, 18.56
Pvu I	6	11.93, 20.82, 21.35, 21.75, 22.47, 33.79
Sac I	2	20.66, 25.09
Sal I	2	30.75, 31.24
Xba I	1	24.82
Xho I	1	31.50

Table 43. Sources for Plasmid Sequence Information

The sequence and location of restriction sites for each plasmid are provided in the sources listed below, available from Promega.

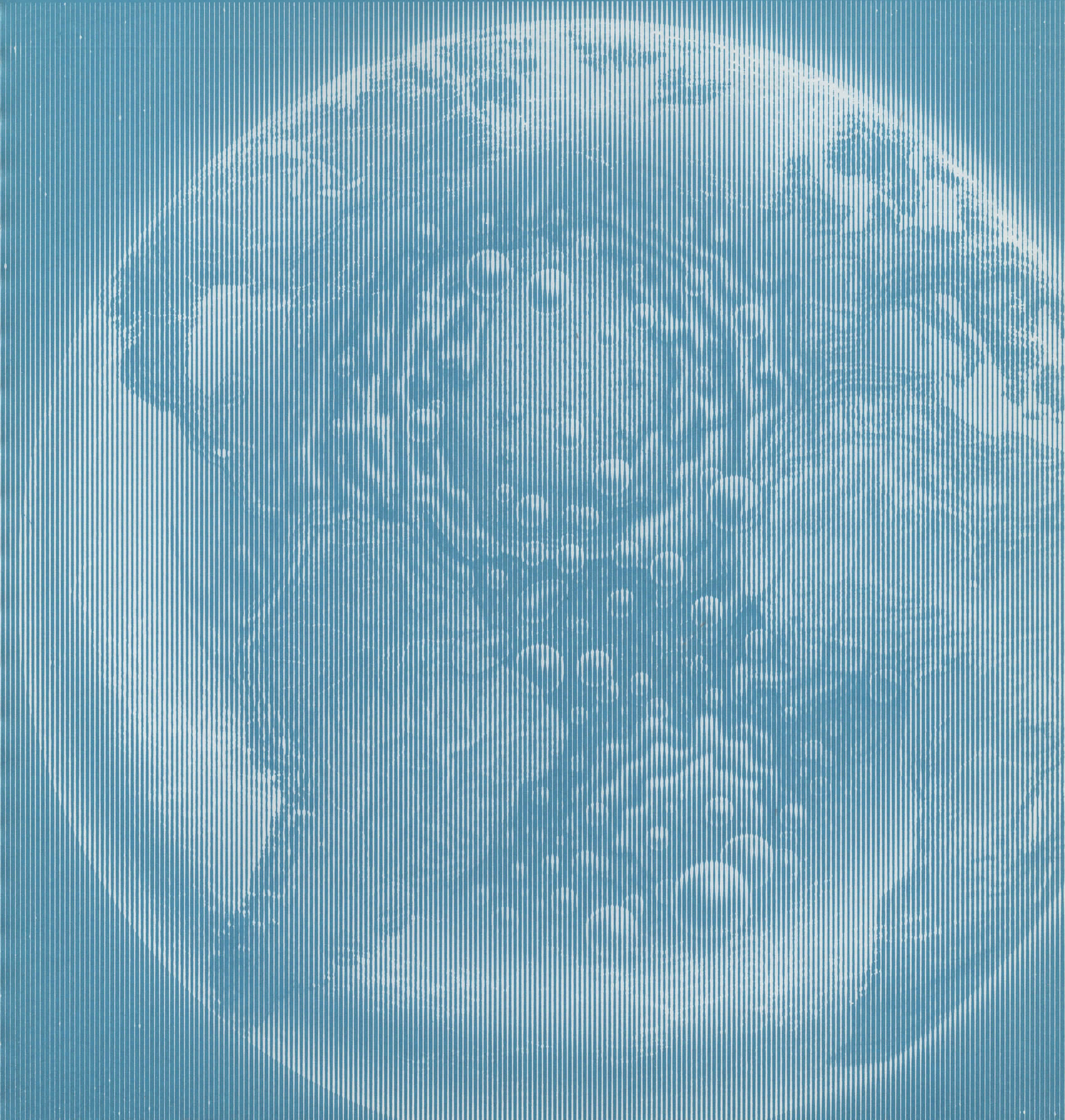
pSP64 vector	Technical Bulletin 012*
pSP65 vector	Technical Bulletin 013*
pSP64 poly(A) vector	Technical Bulletin 052*
pSP70 vector	Technical Bulletin 038*
pSP71 vector	Technical Bulletin 039*
pSP72 vector	Technical Bulletin 040*
pSP73 vector	Technical Bulletin 041*
pGEM®-1 vector	Technical Bulletin 014
pGEM®-2 vector	Technical Bulletin 015*
pGEM®-3 vector	Technical Bulletin 016*
pGEM®-4 vector	Technical Bulletin 017*
pGEM®-3Z vector	Technical Bulletin 033*
pGEM®-4Z vector	Technical Bulletin 036*
pGEM®-3Zf(+) vector	Technical Bulletin 086*
pGEM®-3Zf(–) vector	Technical Bulletin 045*
pGEM®-5Zf(+) vector	Technical Bulletin 047*
pGEM®-5Zf(–) vector	Technical Bulletin 068
pGEM®-7Zf(+) vector	Technical Bulletin 048*
pGEM®-7Zf(–) vector	Technical Bulletin 069
pGEM®-9Zf(–) vector	Technical Bulletin 070*
pGEM®-11Zf(–) vector	Technical Bulletin 074*
pGEM®-11Zf(+) vector	Technical Bulletin 075*
pGEM®-13Zf(+) vector	Technical Bulletin 073*
pGEMEX™-1 vector	Technical Bulletin 078*
pGEMEX™-2 vector	Technical Bulletin 079*
pSELECT™-1 vector	Altered Sites™ *in vitro* Mutagenesis Manual*
pCAT®-Basic vector	Technical Bulletin 080*
pCAT®-Enhancer vector	Technical Bulletin 082*
pCAT®-Promoter vector	Technical Bulletin 083*
pCAT®-Control vector	Technical Bulletin 081*
pSV-β-Galactosidase control plasmid	Technical Bulletin 094*

*The sequences and restriction sites for these vectors are also provided on the pGEM® vector diskette.

Vector Maps

Notes

PROTOCOLS AND APPLICATIONS GUIDE



Technical Appendix

Contents

Technical Appendix

Properties of Nucleic Acids and Proteins

Table 1. Physical Constants of the Nucleoside Triphosphates.

Compound	M.W.	λ_{max} (pH 7.0)	Absorbance at λ_{max} for 1M Solution (E) (pH 7.0)
ATP	507.2	259	15,400
CTP	483.2	271	9,000
GTP	523.2	253	13,700
UTP	484.2	262	10,000
dATP	491.2	259	15,200
dCTP	467.2	271	9,300
dGTP	507.2	253	13,700
dTTP	482.2	267	9,600

Conversion formula

$$\frac{\text{(observed absorbance at } \lambda_{max}\text{)}}{\text{absorbance at } \lambda_{max} \text{ for 1M solution}} = \text{molar concentration of nucleic acid}$$

Reference:

Freifelder, D. (1982) in *Physical Biochemistry*, W.H. Freeman and Company, 494-536.

Table 2. Lengths and Molecular Weights of Common Nucleic Acids.

Nucleic Acid	Number of Nucleotides	Molecular Weight
Lambda DNA	48,502 (circular, dsDNA)	3.0×10^7
pBR322 DNA	4,363 (dsDNA)	2.8×10^6
28S rRNA	4,800	1.6×10^6
23S rRNA	3,700	1.2×10^6
18S rRNA	1,900	6.1×10^5
16S rRNA	1,700	5.5×10^5
5S rRNA	120	3.6×10^4
tRNA (*E. coli*)	75	2.5×10^4

Standards

1. 1kb of double-stranded DNA (sodium salt) = 6.6×10^5 Daltons.
2. 1kb of single-stranded DNA (sodium salt) = 3.3×10^5 Daltons.
3. 1kb of single-stranded RNA (sodium salt) = 3.4×10^5 Daltons.
4. The average MW of a deoxynucleotide base = 324.5 Daltons.

Properties of Nucleic Acids and Proteins

(continued)

Table 3. Common Conversions of Nucleic Acids and Proteins.

I. Weight Conversions

$1\mu g = 10^{-6}g$

$1ng = 10^{-9}g$

$1pg = 10^{-12}g$

$1fg = 10^{-15}g$

II. Spectrophotometric Conversions

$1A_{260}$ unit of double-stranded DNA = 50µg/ml

$1A_{260}$ unit of single-stranded DNA = 33µg/ml

$1A_{260}$ unit of single-stranded RNA = 40µg/ml

III. DNA Molar Conversions

1µg of 1,000bp DNA = 1.52pmole (3.03 pmoles of ends)

1µg of pBR322 DNA = 0.36pmole DNA

1pmole of 1,000bp DNA = 0.66µg

1pmole of pBR322 DNA = 2.8µg

IV. Protein Molar Conversions

100pmoles of 100,000 MW protein = 10µg

100pmoles of 50,000 MW protein = 5µg

100pmoles of 10,000 MW protein = 1µg

V. Protein/DNA Conversions

1kb of DNA = 333 amino acids of coding capacity = 3.7×10^4 MW

10,000 MW protein = 270bp DNA

30,000 MW protein = 810bp DNA

50,000 MW protein = 1.35kb DNA

100,000 MW protein = 2.7kb DNA

Technical Appendix

Properties of Nucleic Acids and Proteins

(continued)

Table 4. Abbreviations and Molecular Weights for Amino Acids.

Amino Acid	Three-letter Abbreviation	One-letter Symbol	Molecular Weight
Alanine	Ala	A	89
Arginine	Arg	R	174
Asparagine	Asn	N	132
Aspartic acid	Asp	D	133
Asparagine or aspartic acid	Asx	B	—
Cysteine	Cys	C	121
Glutamine	Gln	Q	146
Glutamic Acid	Glu	E	147
Glutamine or glutamic acid	Glx	Z	—
Glycine	Gly	G	75
Histidine	His	H	155
Isoleucine	Ile	I	131
Leucine	Leu	L	131
Lysine	Lys	K	146
Methionine	Met	M	149
Phenylalanine	Phe	F	165
Proline	Pro	P	115
Serine	Ser	S	105
Threonine	Thr	T	119
Tryptophan	Trp	W	204
Tyrosine	Tyr	Y	181
Valine	Val	V	117

Properties of Nucleic Acids and Proteins

(continued)

Table 5. Codon Usage Table.

1st Position	2nd Position U	2nd Position C	2nd Position A	2nd Position G	3rd Position
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
U	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
U	UUA Leu	UCA Ser	**UAA** End	**UGA** End	A
U	UUG Leu	UCG Ser	**UAG** End	UGG Trp	G
C	CUU Leu	CUU Pro	CAU His	CGU Arg	U
C	CUC Leu	CCC Pro	CAC His	CGC Arg	C
C	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
C	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
A	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
A	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
A	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
G	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
G	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
G	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

The codons read in the 5′→ 3′ direction.
Termination codons are in bold.

Technical Appendix

Properties of Nucleic Acids and Proteins

(continued)

Table 6. Prokaryotic Suppressors of Nonsense Mutations.

Suppressor Mutation	Codon Recognized	Amino Acid Inserted
*sup*D	amber (UAG)	serine
*sup*E	amber (UAG)	glutamic acid
*sup*F	amber (UAG)	tyrosine
*sup*B	amber (UAG) ochre (UAA)	glutamic acid
*sup*C	amber (UAG) ochre (UAA)	tyrosine

Reference:

Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nucleic Acid and Protein Marker Sizes

Table 7. Lambda Markers (Uncut Lambda DNA is Cat.# D1501).

Marker Sizes (base pairs)

*Eco*R I (Cat.# G1721)	*Hind* III (Cat.# G1711)	*Eco*R I + *Hind* III (Cat.# G1731)	
21,226	23,130	21,227	1,584
7,421	9,416	5,148	1,375
5,804	6,557	4,973	974
5,643	4,361	4,268	831
4,878	2,322	3,530	564
3,530	2,027	2,027	125
	564	1,904	
	125		

Technical Services:
800-356-9526

Nucleic Acid and Protein Marker Sizes

(continued)

Table 8. pGEM® DNA Markers (Cat.# G1741).

Marker Sizes (base pairs):

2,645	460	126
1,605	396	75
1,198	350	65
676	222	51
517	179	36

Table 9. pBR322 DNA Markers (Uncut pBR322 DNA is Cat.# D1511).

Marker Sizes (base pairs):

Alu I		*Hae* III		*Taq* I	*Hinf* I
910	136	587	123	1,444	1,631
659	100	540	104	1,307	517
655	63	504	89	475	506
521	57	458	80	368	396
403	49	434	64	315	344
281	19	267	57	312	298
257	15	234	51	141	221
226	11	213	21		220
		192	18		154
		184	11		75
		124	7		

Nucleic Acid and Protein Marker Sizes

(continued)

Table 10. Bacteriophage ϕX174 DNA Markers (Uncut ϕX174 DNA is Cat.# D1531).

Marker Sizes (base pairs):

Hae III (Cat.# G1761)	*Hin*f I (Cat.# G1751)		*Taq* I
1,353	726	140	2,914
1,078	713	118	1,175
872	553	100	404
603	500	82	327
310	417	66	231
281	413	48	141
271	311	42	87
234	249	40	54
194	200	24	33
118	151		20
72			

Table 11. High Resolution Analytical Marker DNA (Cat.# DG1911).

This high resolution DNA marker set was developed for use with Promega's GenePrint™ DNA typing products. The marker set is visualized by transfer to a nylon membrane and hybridization with a ^{32}P- or digoxigenin-labeled probe available from Promega.

Marker Sizes (base pairs):

14,321	4,822	1,929
12,715	4,324	1,747
11,933	3,976	1,560
9,533	3,675	1,371
8,453	3,194	1,264
7,242	2,804	1,079
6,369	2,582	964
5,687	2,323	702
5,220	2,121	

Nucleic Acid and Protein Marker Sizes

(continued)

Table 12. RNA Markers (Cat.# G3151).

Marker Sizes (bases)	
9,488	1,898
6,225	872
3,911	562
2,800	363

Table 13. Brome Mosaic Virus RNA (Cat.# D1541).

Translation Product Marker Sizes (daltons)	RNA Marker Sizes (bases)
110,000	3,234
97,000	2,865
35,000	2,117
20,000	816
15,000	

Table 14. ProMega-Markers™ for Chromosomal DNA Analysis.

These DNA markers are designed for pulsed-field gel analysis of chromosomal DNA. They are supplied in orange dye-colored tube molds of agarose, making sample loading easier.

Marker Sizes (base pairs)						
Lambda Wild-Type Concatemer Ladder (Cat.# G3011)		Lambda Delta 39 Concatemer Ladder (Cat.# G3031)		Yeast Chromosomal DNA (Cat.# G3021)		*S. pombe* Chromosomal DNA (Cat.# D1951)
970,000	485,000	823,200	392,000	2,000,000	690,000	5,700,000
921,500	436,500	784,000	352,800	1,500,000	610,000	4,700,000
873,000	388,000	744,800	313,600	1,100,000	450,000	3,500,000
824,500	339,500	705,600	274,400	950,000	345,000	
776,000	291,000	666,400	235,200	820,000	270,000	
727,500	242,500	627,200	196,000	790,000	245,000	
679,000	194,000	588,000	156,800	760,000		
630,500	145,500	548,800	117,600			
582,000	97,000	509,600	78,400			
533,500	48,500	470,400	39,200			
		431,200				

Technical Appendix

Nucleic Acid and Protein Marker Sizes

(continued)

Table 15. Protein Molecular Weight Markers.

High-Range Protein Molecular Weight Markers (Cat.# V5251)

Protein	Molecular Weight
Myosin	212,000
β-Galactosidase	116,000
Phosphorylase B	97,400
Bovine Serum Albumin	66,200
Catalase	57,500
Aldolase	40,000

Mid-Range Protein Molecular Weight Markers (Cat.# V5231)

Protein	Molecular Weight
Phosphorylase B	97,400
Bovine Serum Albumin	66,200
Glutamate Dehydrogenase	55,000
Ovalbumin	42,700
Aldolase	40,000
Carbonic Anhydrase	31,000
Soybean Trypsin Inhibitor	21,500
Lysozyme	14,400

Low-Range Protein Molecular Weight Markers (Cat.# V5241)

Protein	Molecular Weight
Carbonic Anhydrase	31,000
Soybean Trypsin Inhibitor Doublet	20,400/19,700*
Horse Heart Myoglobin	16,900
Lysozyme	14,400
Myoglobin (F1)	8,100
Myoglobin (F2)	6,200
Myoglobin (F3)	2,500

*A high resolution gel system described by Schägger and von Jagow (1) is used to resolve polypeptides in this size range. Using this system, soybean trypsin inhibitor migrates as a doublet of apparent molecular weights of 20,400 and 19,700. These values are slightly less than the soybean trypsin inhibitor apparent molecular weight obtained with the Laemmli gel system (2).

References:

1. Schägger, H. and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368.
2. Laemmli, U.K. (1970) *Nature* **277**, 680.

Reagent and Isotope Properties

Table 16. Temperature Dependence of pH for Commonly Used Buffers.

Buffer System	pK_a at 20°C	ΔpK_a/10°C
Mes	6.15	−0.110
Ada	6.60	−0.110
Pipes	6.80	−0.085
Aces	6.90	−0.200
Bes	7.15	−0.160
Mops	7.20	−0.013
Tes	7.50	−0.200
Hepes	7.55	−0.140
Tricine	8.15	−0.210
Tris	8.30	−0.310
Bicine	8.35	−0.180
Glycylglycine	8.40	−0.280

Reference:

Good, N.E. (1986) *Biochemistry* **5**, 467-476.

Table 17. Temperature Dependence of the pH of 50mM Tris-HCl Solutions.

4°C	25°C	37°C
8.1	7.5	7.2
8.2	7.6	7.3
8.3	7.7	7.4
8.4	7.8	7.5
8.5	7.9	7.6
8.6	8.0	7.7
8.7	8.1	7.8
8.8	8.2	7.9
8.9	8.3	8.0
9.0	8.4	8.1
9.1	8.5	8.2
9.2	8.6	8.3
9.3	8.7	8.4
9.4	8.8	8.5

Technical Appendix

Reagent and Isotope Properties

(continued)

Table 18. Preparation of Common Laboratory Solutions.

Solution	Preparation
agarose gel sample buffer (6X)	Dissolve 4g sucrose and 2.5mg of bromophenol blue in a 6ml solution of 10mM Tris-HCl, pH 8.0 and 1mM EDTA (TE buffer). Once dissolved, bring up to a final volume of 10ml with TE buffer. Store at room temperature.
Denhardt's solution (50X)	Dissolve 5g Ficoll, 5g polyvinylpyrrolidone, and 5g of BSA in 300ml H_2O. Once dissolved, bring up to 500ml total with H_2O. Filter and dispense into 25ml aliquots. Store at –20°C.
1M dithiothreitol (DTT)	Dissolve 3.09g of DTT in 20ml of 0.01M sodium acetate, pH 5.2. Sterilize by filtration. Dispense into 1ml aliquots and store at –20°C.
0.5M EDTA (pH 8.0)	Add 186.1g of disodium ethylene diamine tetraacetate $\cdot 2H_2O$ to 800ml of H_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (about 20g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving.
ethidium bromide, 10mg/ml	Add 1g of ethidium bromide to 100ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer to a dark bottle and store at 4°C. **Caution:** Ethidium bromide is a mutagen and is toxic. Wear gloves when working with ethidium bromide solutions and a mask when weighing it out.
phenol (acid)	Dissolve 500g phenol in 500ml 50mM NaOAc. Once dissolved, let phases separate and remove upper aqueous phase. Add 500ml 50mM NaOAc and stir to emulsify. Let phases separate. Repeat procedure until the pH of the upper phase is less than 4.1.
TE-saturated phenol/chloroform	Dissolve 500g phenol in 500ml chloroform. Once dissolved, add 25ml isoamyl alcohol. Then add 1 volume of 10mM Tris-HCl, pH 8.0, 1mM EDTA (TE buffer). Stir to emulsify. Let phases separate. Remove top phase and repeat TE addition. Let phases separate, removing all but 1cm of aqueous phase. Store in a dark bottle at 4°C.
phosphate-buffered saline (PBS)	Dissolve 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, and 0.24g KH_2PO_4 in 800ml distilled H_2O. Adjust to pH 7.4 with HCl. Add H_2O to 1 liter. Dispense into aliquots. Sterilize by autoclaving.
5M potassium acetate	Add 11.5ml of glacial acetic acid and 28.5ml of H_2O to 60ml of 5M potassium acetate. The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

(continued on next page)

Reagent and Isotope Properties

(continued)

Solution	Preparation
SDS gel sample buffer (2X)	Combine 20ml glycerol, 5ml β-mercaptoethanol, 20ml 10% SDS 20mg bromophenol blue, and 25ml 4X stacking gel buffer (6.06g Tris, 4ml 10% SDS, pH to 6.8, bring to 100ml with H_2O). Dissolve and bring to 100ml with H_2O. Store at room temperature.
10% sodium dodecyl sulfate (SDS)	Dissolve 100g of electrophoresis-grade SDS in 900ml of H_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust volume to 1 liter. Dispense into aliquots.
20X SSC	Dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of H_2O. Adjust pH to 7.0 with a few drops of a 10N solution of NaOH. Adjust volume to 1 liter. Dispense into aliquots. Sterilize by autoclaving.
20X SSPE	Dissolve 174g of NaCl, 27.6g of $NaH_2PO_4 \cdot H_2O$, and 7.4g of EDTA in 800ml of H_2O. Adjust pH to 7.4 with NaOH (6.5ml of a 10N solution). Adjust volume to 1 liter. Dispense into aliquots. Sterilize by autoclaving.
trichloroacetic acid (TCA) 100% solution	Add 227ml H_2O to a bottle containing 500g TCA. The solution will be 100% (w/v) TCA.
urea polyacrylamide gel sample buffer	Dissolve 90ml deionized formamide, 10g sucrose, 20mg bromophenol blue, and 20mg xylene cyanol. Bring up to a final volume of 100ml.

Table 19. Physical Properties of Some Beta Emitting Radionuclides.

Radionuclide	Half-life	Specific Activity: Common Values for Compounds (mCi/mmol)	Daughter Nuclide (stable)
tritium	12-43 years	10^2-10^5	helium-3
carbon-14	5,730 years	1-10^2	nitrogen-14
sulphur-35	87.4 days	1-10^6	chlorine-35
phosphorus-33	25.5 days	10-10^4	sulphur-33
chlorine-36	3.01 x 10^5 years	10^{-3}-10^{-1}	argon-36
phosphorus-32	14.3 days	10-10^6	sulphur-32

Technical Appendix

Reagent and Isotope Properties

(continued)

Table 20. Physical Properties of Some Gamma- and X-ray Emitting Radionuclides.

Radionuclide	Half-life	Specific Activity: Common Values for Compounds (mCi/mmol)	Daughter Nuclide (stable)
iodine-131	8.06 days	10^2-10^4	xenon-131
iodine-125	60 days	10^2-10^6	tellurium-125
cobalt-57	270.5 days	10^3-10^5	iron-57
cobalt-58	70.8 days	10^3-10^5	iron-58
selenium-75	120 days	10-10^3	arsenic-75
mercury-197	64.4 hours	10^2-10^4	gold-97
mercury-203	47 days	10-10^3	thallium-203

Properties of RNasin Ribonuclease Inhibitor

Properties of RNasin® ribonuclease inhibitor are listed in Table 21. Recombinant RNasin ribonuclease inhibitor, recently cloned and developed in Promega's laboratories, has been shown to inhibit the activity of RNase A-type enzymes (Table 22) in a variety of organisms, having the same spectrum of inhibition as the natural protein. Promega's recombinant and natural preparations contain no detectable endonuclease activity as determined by incubation of 200 units of inhibitor with supercoiled DNA. No nicking activity is observed after a 120 minute incubation at 37°C as determined by agarose gel electrophoresis.

Since ribonucleases are a class of enzymes known to be active under denaturing conditions, Promega has studied the hypothesis that denaturation of an RNasin ribonuclease inhibitor-RNase complex may release active ribonucleases. This would be critical in RNA capping or transcription *in vitro* reactions where the RNA is purified or analyzed under denaturing conditions. This hypothesis has been investigated by observing the extent of 5S RNA protection which occurs when subjecting the inhibitor-RNase A complex to denaturing conditions. Treatment of the inhibitor-RNase A complex with 7M urea prior to electrophoresis results in 5S RNA degradation. Under these conditions, the inhibitor is presumably denatured and previously complexed RNAse is released. No degradation of 5S RNA occurs when 1% diethylpyrocarbonate (DEPC) is added prior to treatment with 7M urea. This suggests that simultaneous inactivation of the inhibitor and RNAse A occurs with DEPC treatment. We have also found that heating the inhibitor-RNase complex at 65°C for as little as 10 minutes is enough to liberate the active RNase. Therefore, in experiments ultimately requiring intact RNA, care must be taken to avoid the denaturation of RNasin ribonuclease inhibitor and release of active RNase.

Applications: A wide variety of applications demonstrates the versatility of this protein. RNasin ribonuclease inhibitor has been shown to:

- Improve cDNA synthesis (1).
- Improve protein translation performance (2).
- Improve *in vitro* RNA synthesis (3).
- Increase yields and activity of polysomes (2).
- Improve *in vitro* virus replication (4).
- Improve RNA translation in homologous systems (4,5).
- Help prepare RNase-free antibody (6).
- Enhance yields from Riboprobe® system RNA synthesis reactions (7).
- Protect RNA in other applications where RNase has proven to be a problem, such as DNase digestions, RNA capping and reactions using tobacco acid pyrophosphatase (TAP).
- Abolish both angiogenic and ribonucleolytic activities (8).

In addition, recombinant RNasin ribonuclease inhibitor is free of traces of mammalian DNA which is potentially important for RNA amplification studies (9,10).

Properties of RNasin Ribonuclease Inhibitor

(continued)

Concentration: 20-40 units/µl.

Specific Activity: 100 units/µg.

Unit Definition: One unit is defined as the amount of RNasin ribonuclease inhibitor required to inhibit by 50% the activity of 5ng of ribonuclease A (11).

Note: RNasin ribonuclease inhibitor is active over a broad pH range but requires a minimum of 1mM DTT to maintain activity.

References:

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15. Lee, F.S., *et al.* (1988) *Biochemistry* **27**, 8545-8553.
16. Promega, unpublished results (1982).

Table 21. Properties of RNasin Ribonuclease Inhibitor

Activity	Inactivates RNase by binding noncovalently to the molecule
Type of inhibition	Competitive (12)
Binding constant	K_i=4.4 x 10^{14} (12)
Binding ratio with RNase A	1:1 (13)
Molecular weight	51,000 daltons (13)
Isoelectric point	pH 4.7 (14)
Free sulfhydryl groups	30 (15)
pH activity range	pH 5-8 (highest activity pH 7-8) (13,16)

Table 22. Effectiveness of Recombinant RNase Inhibitor Against Selected Nucleases.

Inhibits	Does Not Inhibit
RNase A	RNase T1
RNase B	S1 Nuclease
RNase C	RNase from Aspergillus

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Abbreviations and Symbols

Table 23. Commonly Used Abbreviations and Symbols.

α	alpha	kb	kilobase
A	adenine or adenosine	kDa	kilodalton
ADP	adenosine diphosphate	λ	lambda
Ad-2	adenovirus	l	liter
AMP	adenosine monophosphate	leu	leucine
AP	alkaline phosphatase	M	molar
ATP	adenosine triphosphate	met	methionine
β	beta	mg	milligram
BCIP	5-bromo-4-chloro-3-indolyl phosphate	Mg^{2+}	magnesium
BMV	brome mosaic virus	$MgCl_2$	magnesium chloride
bp	base pairs	ml	milliliter
BSA	bovine serum albumin	mm	millimeter
C	cytosine or cytidine	mM	millimolar
Ca^{2+}	calcium	Mn^{2+}	manganese
CAT	chloramphenicol acetyltransferase	mRNA	messenger RNA
$CaCl_2$	calcium chloride	MW	molecular weight
cDNA	complementary DNA	NaCl	sodium chloride
CIAP	alkaline phosphatase, calf intestine	NaOAc	sodium acetate
cm	centimeter	NBT	nitro blue tetrazolium
Co^{2+}	cobalt	nmole	nanomole
CNBr	cyanogen bromide	-OH	hydroxyl group
cpm	counts per minute	ONPG	o-nitrophenyl-β-D-galactosidase
CTP	cytosine triphosphate	-P	phosphate group
DNA	deoxyribonucleic acid	PAGE	polyacrylamide gel electrophoresis
DNase	deoxyribonuclease	pg	picogram
DTT	dithiothreitol	pmole	picomole
EDTA	ethylenediamine tetraacetic acid	Pi	inorganic phosphate
EtOH	ethanol	PMSF	phenyl methyl sulfonyl fluoride
γ	gamma	r–	restriction minus
g	gram	RNA	ribonucleic acid
G	guanine or guanosine	RNase	ribonuclease
GTP	guanosine triphosphate	rRNA	ribosomal RNA
^3H	tritium	SAM	S-adenosylmethionine
HCl	hydrochloric acid	SDS	sodium dodecyl sulfate
IPTG	β-D-isopropyl-thiogalactopyranoside	T	thymine or thymidine
KCl	potassium chloride	TAP	tobacco acid pyrophosphatase

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Abbreviations and Symbols

(continued)

TCA	trichloroacetic acid
TE	Tris-EDTA buffer
tRNA	transfer RNA
u	unit
U	uracil or uridine
µg	microgram
µl	microliter
µm	micromolar
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
X-Gal	5-bromo-4-chloro-3-indolyl-β galactopyranoside
$ZnCl_2$	zinc chloride

Table 24. The Greek Alphabet.

Α	α	alpha	Ν	ν	nu
Β	β	beta	Ξ	ξ	xi
Γ	γ	gamma	Ο	ο	omicron
Δ	δ	delta	Π	π	pi
Ε	ε	epsilon	Ρ	ρ	rho
Ζ	ζ	zeta	Σ	σ	sigma
Η	η	eta	Τ	τ	tau
Θ	θ	theta	Υ	υ	upsilon
Ι	ι	iota	Φ	ϕ	phi
Κ	κ	kappa	Χ	χ	chi
Λ	λ	lambda	Ψ	ψ	psi
Μ	μ	mu	Ω	ω	omega

Bacterial Strains and Mutations

Table 25. Antibiotics: Mode of Action and Mechanism of Resistance.

Antibiotics	Mode of Action	Mechanism of Resistance
Ampicillin (Ap)	A derivative of penicillin that kills growing cells by interfering with a terminal reaction in bacterial cell wall synthesis.	The resistance gene (*bla*) specifies a periplasmic enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.
Chloramphenicol (Cm)	A bacteriostatic agent that interferes with bacterial protein synthesis by binding to the 50S subunit of ribosomes and preventing peptide bond formation.	The resistance gene (*cat*) specifies an acetyltransferase that acetylates and thereby inactivates the antibiotic.
Colicin E1 (ColE1)	A member of the general class of substances known as bacteriocins, which are lethal for strains of bacteria not carrying a resistance gene; colicin E1 effects lethal membrane changes in target bacteria.	The resistance gene (*cea*) specifies a product that interferes with the action of the colicin in an unknown fashion.
Kanamycin (Km)	A bacteriocidal agent that binds to 70S ribosomes and causes misreading of messenger RNA.	The resistance gene (*kan*) specifies an enzyme that modifies the antibiotic and prevents its interaction with ribosomes.
Streptomycin (Sm)	A bacteriocidal agent that binds to the 30S subunit of ribosomes and causes misreading of the messenger RNA.	The resistance gene (*str*) specifies an enzyme that modifies the antibiotic and inhibits its binding to the ribosome.
Tetracycline (Tc)	A bacteriostatic agent that prevents bacterial protein synthesis by binding to the 30S subunit of ribosomes.	The resistance gene (*tet*) specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.

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(continued)

Table 26. Genotypes of Frequently Used Bacterial Strains.

The genes listed for each strain indicate mutant alleles carried by that bacterium. Genes listed on the F′ episome, however, represent wild type alleles unless specified otherwise. Strains are λ^- unless specified otherwise.

* Strains available from Promega as competent cells are indicated by an asterisk.

Strains shown in **bold** are available from Promega as glycerol freezer stocks.

Strain	Genotype
BMH 71-18 mut S	*thi*, *sup*E, Δ(*lac-pro*AB), [*mut*S::Tn10] [F′, *pro*AB, *laqI*^{q}ZΔM15]
C600 (1)	F$^-$, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44
C600*hfl* (1)	F$^-$, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44, *hfl*A150, [*chr*::Tn10]
DH1 (2)	F$^-$, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1
DH5α	F$^-$, φ80d*lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169
DH5αF′	F′, φ80d *lac*ZΔM15, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_K^-, m_K^+), *sup*E44, *rel*A1, *deo*R, Δ(*lac*ZYA-*arg*F)U169
***HB101** (3)	F$^-$, *thi*-1, *hsd*S20 (r_B^-, m_B^-), *sup*E44, *rec*A13, *ara*-14, *leu*B6, *pro*A2, *lac*Y1, *rps*L20 (str^r), *xyl*-5, *mtl*-1
JM83 (4)	F$^-$, *ara*, Δ(*lac-pro*AB), *rps*L, φ80d*lac*ZΔM15
JM101 (4)	*sup*E, *thi*, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
JM103 (2)	*end*A1, *hsd*R, *sup*E, *sbc*B15, *thi*-1, *str*A, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
JM105 (4)	*end*A1, *thi*, *rps*L, *sbc*B15, *hsd*R4, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
JM107 (4)	*end*A1, *thi*, *gyr*A96, *hsd*R17 (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
JM108 (4)	F$^-$, *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB)
***JM109** (4)	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
JM109 (DE3) (4)	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_K^-, m_K^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15], λ(DE3)
JM110 (4)	*rps*L, *thr*, *leu*, *thi*, *hsd*R17 (r_K^-, m_K^+), *lac*Y, *gal*K, *gal*T, *ara*, *ton*A, *tsx*, *dam*, *dcm*, *sup*E44, Δ(*lac-pro*AB), [F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔM15]
LE392 (5)	F$^-$, *hsd*R574, (r_K^-, m_K^+), *sup*E44, *sup*F58, *lac*Y1 or Δ(*lac*IZY)6, *gal*K2, *gal*T22, *met*B1, *trp*R55
KW251	F$^-$, *sup*E44, *gal*K2, *gal*T22, *met*B1, *hsd*R2, *mcr*B1, *mcr*A, [*arg*A81:Tn10], *rec*D1014
***MB408** (6)	F$^-$, *rec*F, *rec*B21, *rec*C22, *sbc*B15, *hfl*A, *hfl*B, *hsd*R, (tet^r)
NM522 (7)	F$^-$, *sup*E, *thi*, Δ(*lac-pro*AB), Δ*hsd*5 (r^-, m^-), [F′, *pro*AB, *lac*I^{q}ZΔM15]
NM538 (8)	F$^-$, *sup*F, *hsd*R (r_K^-, m_K^+), *trp*R, *lac*Y
NM539 (8)	F$^-$, *sup*F, *hsd*R (r_K^-, m_K^+), *lac*Y, (P2)
RR1 (9)	F$^-$, *hsd*S20, (r_B^-, m_B^+), *sup*E44, *ara*-14, *pro*A2, *rsp*L20 (str^r), *lac*Y1, *gal*K2, *xyl*-5, *mtl*-1, *sup*E44
X1776 (9)	F$^-$, *ton*A53, *dap*D8, *min*A1, *gln*V44, (*sup*E44), Δ(*gal-uvr*B)40, *min*82, *rib*-2, *gyr*A25, *thy*A142, *oms*-2, *met*C65, *oms*-1, (*tte*-1), Δ(*bio*H-*asd*)29, *cyc*82, *cyc*A1, *hsd*R2

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(continued)

Strain	Genotype
Y1088 (10)	F′, Δ(*lac*U169), *sup*E, *sup*F, *hsd*R (r^-, m^+), *met*B, *trp*R, *ton*A21, [*pro*C::Tn5] (pMC9)
Y1089 (10)	F′, Δ(*lac*U169), *pro*A$^+$, Δ(lon), *ara*D139, *str*A, *hfl*A150, [chr:Tn10(tet^r)], (pMC9)
Y1090 (10)	F′, Δ(*lac*U169), *pro*A$^+$, Δ(lon), *ara*D139, *str*A, *sup*F, [*trp*C22::Tn10(tet^r)], (pMC9), *hsd*R(r_K^-, m_K^+)

References:

1. Jendrisak, J., Young, R.A. and Engel, J. (1978) In: *Guide to Molecular Cloning Techniques*, Eds. Berger, S. and Kimmel, A., Academic Press, 359-371.
2. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557-580.
3. Lacks, S. and Greenberg, J.R. (1977) *J. Mol. Biol.* **114**, 153.
4. Yanisch-Perron, C., Viera, J. and Messing, J. (1985) *Gene*, **33**, 103-119.
5. Murray, N. (1977) *Mol. Gen. Genet.* **150**, 53-58.
6. Schatz, D., *et al.* (1989) *Cell* **59**, 1035.
7. Gough, J. and Murray, N. (1983) *J. Mol. Biol.* **166**, 1-19.
8. Frischauf, A., *et al.* (1983) *J. Mol. Biol.* **170**, 827-842.
9. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
10. Huynh, T., Young, R.A. and Davis, R. (1985) In: *DNA Cloning*, Vol. 1, Ed. Glover, D., IRL Press Ltd, 56-110.

Table 27. Genetic Markers in Frequently Used *E. coli* Strains.

Symbol	Description	Effect
ara-14	Mutation in arabinose metabolism	Blocks arabinose catabolism.
dam	Adenine methylase mutation	Blocks methylation of adenine residues in the sequence 5′...G^{m}ATC...3′.
dcm	Cytosine methylase mutation	Blocks methylation of cytosine in the sequence 5′...C^{m}CAGG...3′ or 5′...C^{m}CTGG...3′.
*deo*R	Regulatory gene mutation allowing constitutive expression of genes for deoxyribose synthesis	Allows uptake of large plasmids.
*end*A1	Endonuclease mutation	Improves quality of plasmid DNA isolations.
*gal*K	Galactokinase mutation	Blocks catabolism of galactose.
*gyr*A96	DNA gyrase mutation	Confers resistance to nalidixic acid.
*hfl*A150 (1)	Mutation leading to a high frequency of lysogeny	Leads to greatly enhanced frequency of lysogeny in λ phages containing a normal repressor (cI) gene.

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Table 27. Genetic Markers in Frequently Used *E. coli* Strains.

Symbol	Description	Effect
*hsd*R (r_K^-,m_K^+)	Restriction minus, modification positive	Allows cloning without cleavage of DNA by endogenous restriction endonucleases. DNA prepared from this strain can be used to transform r_K^+ *E. coli* strains.
*hsd*S20 (r_B^-,m_B^-)	Restriction minus, modification minus	Allows cloning without cleavage of DNA by endogenous restriction endonucleases. DNA prepared from this strain is unmethylated by the *hsd*S20 methylases.
*lac*I^q	Overproduction of the *lac* repressor protein	Leads to high levels of the *lac* repressor protein, inhibiting transcription from the *lac* promoter.
*lac*Y	Galactoside permease mutation	Blocks lactose utilization.
*lac*Y1	β-D-galactosidase mutation	Blocks lactose utilization.
*lac*ZΔM15	Partial deletion of β-D-galactosidase gene	Allows complementation of β-galactosidase activity by α-complementation sequence in pGEM®-Z vectors. Allows blue/white selection for recombinant colonies when plated on X- gal.
*leu*B	β-isopropyl malate dehydrogenase mutation	Requires leucine for growth on minimal media.
Δ(*lon*)	Deletion of *lon* protease	Reduces proteolysis of expressed fusion proteins.
*mcr*A	Mutation in restriction system	Blocks restriction of DNA methylated at the sequence 5′..G^{m}CGC..3′.
*mcr*B	Mutation in restriction system	Blocks restriction of DNA methylated at the sequence 5′..AG^{m}CT..3′.
*met*B	Cystathionine γ-synthase mutation	Requires methionine for growth on minimal media.
mtl-1	Mutation in mannitol metabolism	Blocks catabolism of mannitol.
P2 (2)	P2 bacteriophage lysogen present in host	λ phages containing the *red* and *gam* genes of λ are growth inhibited by P2 lysogens.
*pro*AB	Mutations in proline metabolism	Requires proline for growth in minimal media.

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(continued)

Symbol	Description	Effect
*rec*A1, *rec*A13	Mutation in recombination	Prevents recombination of introduced DNA with host DNA, ensuring stability of inserts. Inserts are more stable in *rec*A1 than *rec*A13 hosts.
*rel*A	Relaxed phenotype; mutation eliminating stringent factor	Allows RNA synthesis in the absence of protein synthesis.
*rec*B, *rec*C	Exonuclease V mutations	Reduces general recombination and affects repair of radiation damage.
*rps*L	Mutation in subunit S12 of 30S ribosome	Confers resistance to streptomycin.
*sbc*B	Exonuclease I mutation	Allows general recombination in *rec*BC mutant strains.
*sup*B, *sup*C, *sup*G, *sup*L, *sup*M, *sup*N, *sup*O	Suppressor mutations	Suppress ochre (UAA) and amber (UAG) mutations.
*sup*D, *sup*E, *sup*F	Suppressor mutations	Suppress amber (UAG) mutations.
thi-1	Mutation in thiamine metabolism	Thiamine required for growth in minimal media.
*Tn*5	Transposon	Encodes resistance to kanamycin.
*Tn*10	Transposon	Encodes resistance to tetracycline.
*ton*A	Mutation in outer membrane protein	Confers resistance to bacteriophage T1.
*tra*D36	Transfer factor mutation	Prevents transfer of F′ episome.
xyl-5	Mutation in xylose metabolism	Blocks catabolism of xylose.

Miscellaneous:

F^-	Host does not contain an F′ episome.
F′	Host contains an F′ episome with the stated features.
λ(DE3)	Bacteriophage λ carrying the gene for T7 RNA polymerase is integrated into the host genome.

References:

1. Hoyt, A. (1982) *Cell* **31**, 565-569.
2. Kaiser, K. and Murray, N. In: *DNA Cloning* Vol.1, Eds. Glover D., IRL Press Ltd. 1-47.

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Descriptions of Bacterial Strains Supplied by Promega

HB101

HB101 has long been a popular strain for propagating pBR322 and other plasmids that do not allow α-complementation. It is an *E. coli* K strain with the *hsd* locus, *mcr*B locus and the *mrr* locus from an *E. coli* B strain. This results in a restriction minus (*hsd*S20) background and partial elimination of the systems which restrict methylated cytosine and adenine residues (*mcr*B, *mrr*). This strain contains an amber suppressor and is *rec*A⁻, therefore eliminating homologous recombination. HB101 is also streptomycin resistant. HB101 high efficiency competent cells are available from Promega.

Reference:

Lacks, S. and Greenberg, J.R. (1977) *J. Mol. Biol.* **114**, 153.

JM109

JM109 is a useful host in which to clone pGEM® plasmids and for production of single-stranded DNA from M13 or phagemid vectors. The strain grows well and is efficiently transformed by a variety of methods (pg. 52-54). Because JM109 is *rec*A⁻ and lacks the *E. coli* K restriction system, undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The endonuclease A⁻ mutation leads to an improved yield and quality of isolated plasmid DNA.

JM109 can be used for blue/white color screening of the pGEM-Z and pGEM-Zf plasmids. JM109 is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the *lac*Z gene. The deletion in the episomal (F factor) copy of the *lac*Z gene (*lac*ZΔM15) is located in the α-peptide region and, as a result, β-galactosidase activity can be complemented by addition of a functional α-peptide. The pGEM-Z and pGEM-Zf vectors encode the *lac*Z α-peptide and cells carrying these plasmids are able to produce functional β-galactosidase. When plated on indicator media containing X-Gal and IPTG, this host/plasmid combination will generate blue colonies. However, when the α-peptide is disrupted by cloning into the multiple cloning region of the pGEM-Z or pGEM-Zf vector, complementation does not occur and no β-galactosidase activity is produced. Therefore, bacterial colonies harboring recombinant pGEM-Z vector constructs remain white.

JM109 should always be maintained on minimal plates (M-9) supplemented with 1mM thiamine-HCl. This selects for the presence of the F′ which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positives. **Note:** For some plasmids, such as the pGEM-3Z and pGEM-4Z plasmids, colonies containing β-galactosidase activity grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies are pinpoint in size while the white colonies are approximately a millimeter in size. JM109 high efficiency competent cells are available from Promega.

Reference:

Hanahan, D. (1985) In: *DNA Cloning Volume 1*, ed. D. Glover, IRL Press Ltd. 109-135.

JM109(DE3)

JM109(DE3), derived from JM109, contains a chromosomal copy of the gene for T7 RNA polymerase. JM109(DE3) is used for the high-level expression of genes cloned into the pGEMEX™-1 or pGEMEX-2 vectors. In these vectors, cloned DNA is inserted downstream from a T7 promoter and is expressed as a fusion protein with a 260 amino acid leader peptide. JM109(DE3) cells can also be used as a host for other pGEM plasmids for expression of sequences downstream from the T7 promoter, provided that the cloned sequence contains a ribosomal binding site.

Descriptions of Bacterial Strains Supplied by Promega

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BMH 71-18 mut S

BMH 17-18 mut S is a highly transformable mismatch repair minus strain of *E. coli.* Use of this strain prevents repair of the newly synthesized unmethylated strand (1,2), leading to high mutation efficiencies. BMH 71-18 mut S is *rec*A$^+$ and, as a result, inserts containing highly repetitive sequences may be unstable.

The BMH 71-18 mut S strain can be used for blue/white screening of the pSELECT™-1 and pSELECT-Control vectors as described for the JM109 strain.

BMH 71-18 mut S should always be maintained on minimal plates (M-9) supplemented with 1mM thiamine-HCl. This selects for the presence of the F′ which carries a nutritional requirement for growth (proline biosynthesis) and decreases the number of false positives.

References:

1. Miller, J. (1972) In: *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 201-205.

2. Kramer, B., Kramer, W. and Fritz, H.-J. (1984) *Cell* **38**, 879.

KW251

The bacterial strain KW251 is a permissive host strain, used as an alternative host to LE392 for genomic cloning. KW251 lacks the *E. coli* K restriction system and is also *rec*D$^-$. Certain eukaryotic clones are unable to grow on, and genomic libraries can be biased by, *rec*$^+$ host strains such as NM538, NM539, and LE392. If low cloning efficiencies are noted using conventional host strains, KW251 may be substituted. In addition, KW251 is *mcr*A$^-$ and *mcr*B$^-$, allowing the propagation of genomic DNA clones containing methylated cytosine residues. KW251 should be maintained in the presence of 15µg/ml tetracycline.

MB408

The MB408 strain allows propagation of clones containing palindromic sequences. This strain contains *rec*B, *rec*C, *rec*F, and *sbc* mutations which totally suppresses recombination and inactivates exonucleases I and V, allowing stable propagation of clones with direct and inverted DNA repeats in *red*$^-$ lambda vectors. Two *hfl* (high frequency of lysogeny) mutations make MB408 an excellent selective host for *c*I insertion vectors such as λgt10. This strain is also restriction minus, modification plus (*hsd*R$^-$) and tetracycline resistant. MB408 competent cells are available from Promega.

Reference:

Dove, W.F., *et al.* (1986) *The Molecular Biology of Physarum Polycephalum.* Plenum Press, New York, pg. 297.

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Descriptions of Bacterial Strains Supplied by Promega

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NM538 and NM539

The NM538 and NM539 host strains are provided with the EMBL3, EMBL4, LambdaGEM®-11 and LambdaGEM-12 genomic cloning vectors when these vectors are supplied as uncut DNA. The host NM538 is a permissive strain, allowing both parental and recombinant phage to grow. NM539 is a restrictive host (a P2 strain), used for *Spi* selection of recombinant phage.

Spi (sensitivity to P2 interference) selection is based on the fact that phage replication is inhibited in P2 lysogens when the phage contains functional *red* and *gam* genes (present on the stuffer fragment of the lambda cloning vector). When this stuffer DNA is replaced by the DNA insert in a recombinant phage, replication of the phage can proceed.

A significant disadvantage of *Spi* selection is that *rec*BC$^+$ hosts must be used, leading to the deletion or rearrangement of potential clones by recombination and the generation of biased genomic libraries. This problem can be overcome by the use of *Bam*H I or *Xho* I half-site arms of the LambdaGEM-11 and LambdaGEM-12 vectors. The extremely low background of nonrecombinants obtained with these arms eliminates the need for *Spi* selection, allowing the use of a *rec*$^-$ strain such as KW251. A further disadvantage of the NM538 and NM539 strains is that lambda plaques in these strains are smaller and thus harder to screen than plaques formed on the KW251 or LE392 strains.

Reference:

Frischauf, A., *et al.* (1983) *J. Mol. Biol.* **170**, 827.

NM522

NM522 is supplied with the pGEM® single strand systems for the production of cloned inserts as single-stranded DNA (ssDNA). This strain is restriction$^-$, *rec*$^+$, and contains an F′ episome. The F′ is required for production of ssDNA and for blue/white color selection (see JM109 stain description). To maintain the F′, NM522 should be grown on minimal (M-9) plates.

Reference:

Gough, J. and Murray, N. (1983) *J. Mol. Biol.* **166**, 1.

LE392

LE392 is used for both genomic and cDNA cloning. LE392 lacks the *E. coli* K restriction system but is *rec*$^+$. It is a permissive host, allowing both recombinant and parental phage to grow. Selection against parental phage is not necessary, however, when cloning into the pre-digested *Bam*H I or *Xho* I half-site arms of the EMBL3, EMBL4, LambdaGEM®-11, or LambdaGEM®-12 genomic cloning vectors.

LE392 is provided with the lambda expression vectors λgt11, λgt11 *Sfi-Not*, and λgt22 as an alternative host to Y1090. Unlike Y1090, LE392 does not allow color selection of recombinants or IPTG induction of expression. Also, since LE392 contains *lon* protease activity, fusion proteins are often less stable in this host than in Y1090. However, due to the minimal background of religated vector arms*, color selection is not necessary to distinguish recombinants. When using this strain, phage titers also tend to be higher than with Y1090 host cells. Therefore, we recommend (if color selection is not required) that LE392 be the primary strain for amplification of recombinant phage and for screening a cDNA library with nucleic acid probes.

*Typical background levels of nonrecombinant phage range from 1-5 x 10^4pfu/µg of arms. Typical recombinant efficiencies range from 5-9 x 10^6pfu/µg arms.

Reference:

Murray, N. (1977) *Mol. Gen. Genet.* **150**, 53.

Descriptions of Bacterial Strains Supplied by Promega

(continued)

C600 and C600*hfl*

The C600 and C600*hfl* strains are provided with the λgt10 cDNA cloning vector. In this vector, DNA is cloned into a unique *Eco*R I site in the *c*I gene, leading to inactivation of the lambda repressor. Recombinant phage are therefore lytic and produce clear plaques, whereas parental phage are lysogenic and produce turbid plaques. C600 is a permissive host, allowing both parental and recombinant phage to grow. C600*hfl* is a restrictive host, allowing recombinant (lytic) phage to grow in preference to parental phage. The high frequency of lysogeny mutation (*hfl*A150) in C600*hfl* allows cI$^+$ (parental) phage to be repressed 50 to 100-fold. Thus, parental phage are minimized in cDNA libraries grown in the C600*hfl* strain. C600*hfl* should be maintained in the presence of 15μg/ml tetracycline.

Reference:

Huynh, T., Young, R.A., and Davis, R. (1985) In: *DNA Cloning Volume 1*, Ed. D. Glover, IRL Press Ltd. 56-110.

Y1090

The *E. coli* host Y1090 has been converted to a restriction minus (r^-m^+) strain by Promega so that cDNA inserts will not be subject to host restriction digestion. Y1090 has three features that are useful for screening expression libraries with antibody probes.

1. The Y1090 bacterial strain is deficient in the *lon* protease. In *lon*$^-$ cells, β-galactosidase fusion proteins often accumulate to much higher levels than in wild-type cells.
2. The Y1090 strain is *sup*F, suppressing the normally defective lysis of λgt11, λgt11 *Sfi-Not*, and λgt22 and leading to a high frequency of lytic plaques. This facilitates antibody screening in two ways. Plaque lifts are simpler and offer better signal to noise ratios than colony lifts. Also, plaques can be screened at a higher density (1-5 x 10^5/plate) than can lysogenic colonies (5-10 x 10^3/plate).
3. The Y1090 strain also contains the plasmid pMC9 (Amp^r, Tet^r). A derivative of pBR322, the plasmid contains the *lac*I gene which encodes the *lac* repressor. This allows the regulated expression of β-galactosidase fusion proteins. Expression of foreign proteins, some of which may. be harmful to growth of the bacterial host, is repressed during initial growth of the phage but is expressed later after induction with isopropyl-β-D-thiogalactoside (IPTG). To maintain the pMC9 plasmid, it is necessary to initially streak this strain on LB plates supplemented with 100μg/ml ampicillin and 15μg/ml tetracycline. All subsequent growth can be in the absence of ampicillin.

The Δ(*lac*U169) deletion present in Y1090 prevents the generation of endogenous functional β-galactosidase protein products. In Y1090, parental phage containing an intact *lac*Z gene produce blue plaques in the presence of X-Gal. Recombinant phage containing a cDNA insert within the *lac*Z gene are not able to compensate for the host mutation and thus generate clear plaques in the presence of X-Gal.

Reference:

Huynh, T., Young, R.A., and Davis, R. (1985) In: *DNA Cloning Volume 1*, Eds. Glover, D., IRL Press Ltd. 56-110.

Y1089

Y1089 is used primarily for the generation of preparative amounts of recombinant fusion protein. This is possible because of a mutation which enhances the frequency of phage lysogeny (*hfl*A150) and the Δ*lon* mutation which decreases protease activity. A recombinant cDNA library is first screened in the bacterial strain Y1090. Once the desired clone is detected, the recombinant phage is plaque-purified and used to infect strain Y1089. After the Y1089 lysogen is grown to high cell density, the *lac*Z-directed fusion protein is induced by the addition of IPTG to the medium and the cells are harvested and lysed. The Y1089 strain should be maintained on LB plates supplemented with 100μg/ml ampicillin and 15μg/ml tetracycline.

Reference:

Huynh, T., Young, R.A., and Davis, R. (1985) In: *DNA Cloning Volume 1*, Eds. Glover, D., IRL Press Ltd. 56-110.

Technical Appendix

Notes

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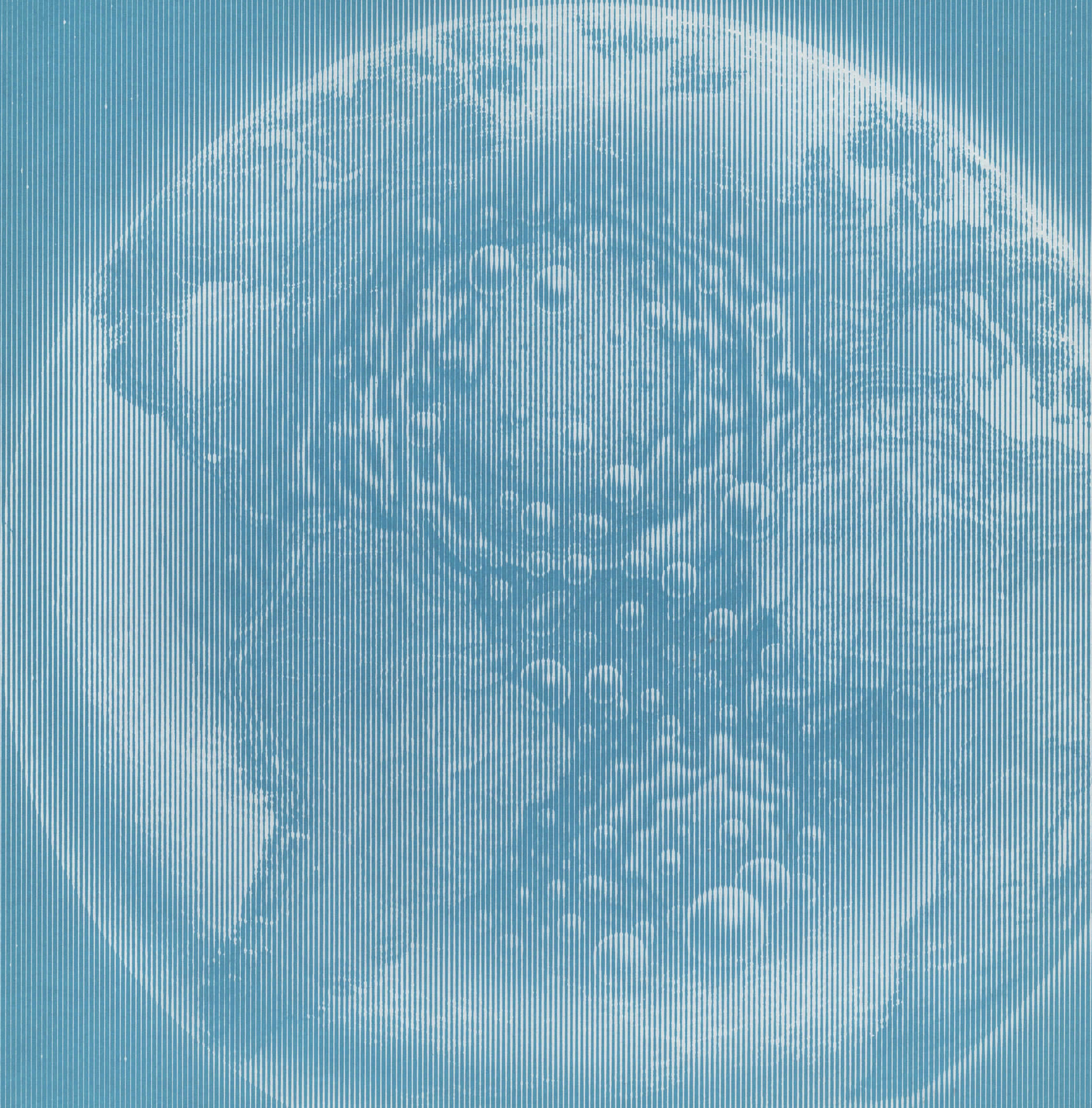

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